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THE FUJINAMI MYXOSARCOMA IN DUCKS: RESISTANCE TO REINFECTION.

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ADULT ducks are readily infected by the agent of the Fujinami myxosarcoma (Gye, 1931), but the infection is rarely fatal. When a massive dose of infective material is injected—2·0 c.c. of minced tumour tissue, for example—the duck may be overwhelmed and killed (Purdy, 1932), but when more ordinary doses are used, recovery is to be expected. After recovery—that is, when the tumour which develops as a result of the inoculation has retrogressed and disappeared—the duck is completely resistant to re-infection by the Fujinami agent (Gye, 1932); even massive doses of minced tissue now fail to produce palpable tumours. This tendency to spontaneous cure and this resistance to a second infection are notable features associated with the Fujinami myxosarcoma as it occurs in ducks. It is to the resistance to re-infection that attention is particularly directed in the present paper. A series of experiments has been carried out with the primary object of finding when the refractory state first becomes detectable in tumour-bearing ducks. Representatives of these experiments will be described and discussed.

All ducks used in the experiments were adults, and all were of the Khaki Campbell variety. Ducks selected for any one experiment were all closely similar to one another in age and weight; and particular care was taken to divide them into groups which should be as nearly alike as possible.

The site of inoculation varied. Sometimes the infective material was injected into the muscles of the leg and sometimes into the wing membrane. In the leg small tumours cannot be recognized and palpated with accuracy, since they lie deeply buried in a mass of muscle. The wing membrane—patagium—which stretches between the humerus and radius is a more suitable site when accurate clinical observations are highly desirable. If the feathers have been removed from the under-surface of this membrane by means of a

barium sulphide preparation, and if the inoculum has been placed just beneath the skin at a point which is not immediately adjacent to the limb muscles, then the developing tumours will be both visible and palpable from the earliest stages. The subcutaneous tissue of the under-surface of the wing membrane is close in texture. In it tumours grow as well-defined nodules, and may reach very large size—100 c.c. for example—without ulceration or physical damage. Unlike fowls, ducks do not peck their wing tumours.

The infectious material has varied. Sometimes cell-free extracts have been injected into the ducks, and sometimes tissue mince which has been pumped free from entangled bubbles of air. Mince has been preferred for the first of two successive inocula, no matter what was to be used for the second. Mince is usually potent, whilst filtrate is apt to prove impotent or only slightly active. Mince, too, gives comparative certainty of the times at which the new tumours will appear and run through their various stages of growth and retrogression. It may be remarked also that when two consecutive inocula have been given to a duck, care has been taken that if the first has consisted of material taken from a duck, then the second has consisted of material taken from a fowl, and *vice versâ*. Perhaps this precaution was unnecessary. It was adopted to exclude effects of species-specific reactions provoked by tissue elements which chanced to accompany the tumour virus.*

The first experiments to which attention is drawn are those summarized in Fig. 1. These were designed to show whether, three days after receiving potent material derived from a Fujinami myxosarcoma, a duck will still react in a normal way to another dose of potent material. All three experiments were carried out on one general plan, and this plan may, perhaps, be described most easily if for the moment attention is restricted to Experiment 1. In this experiment Ducks 3 and 4 each received two injections of minced Fujinami myxosarcoma, the interval, as already explained, being three days. Two controls were necessary, one to exclude the possibility that Ducks 3 and 4 were not normal birds at the beginning of the experiment, and another to show the effect to be expected from the second inoculations in Ducks 3 and 4 provided these birds remained normal. The first control was provided by Ducks 1 and 2, and the second by Ducks 5 and 6. Study of the growth curves of Experiment 1, given in Fig. 1, will show that Ducks 3 and 4 were indeed approximately normal at the beginning of the experiment; the tumours developing in these birds as the result of the first injections were not widely different from those developing in the control Ducks 1 and 2, which received similar doses of the same infective material. The similarity of the two sets of tumours indicates, too, that in Ducks 3 and 4 the tumours developing from the second inocula did not materially affect the growth of the tumours developing from the first. Had it been otherwise interpretation of the results would have been more difficult. In contrast with this similarity are the differences observed in the results of the second set of injections. The growth-curves show that the tumours developing in Ducks 3 and 4 as the result of the second inoculations

* It seems advisable, as here, to reserve the term "virus" to indicate the living organism itself, and to use the term "agent" in the sense in which it was originally used by Rous, viz. to connote the complete infective complex of which, as is now known (Gye and Purdy, 1931), the virus is only one of several essential co-existent constituents.

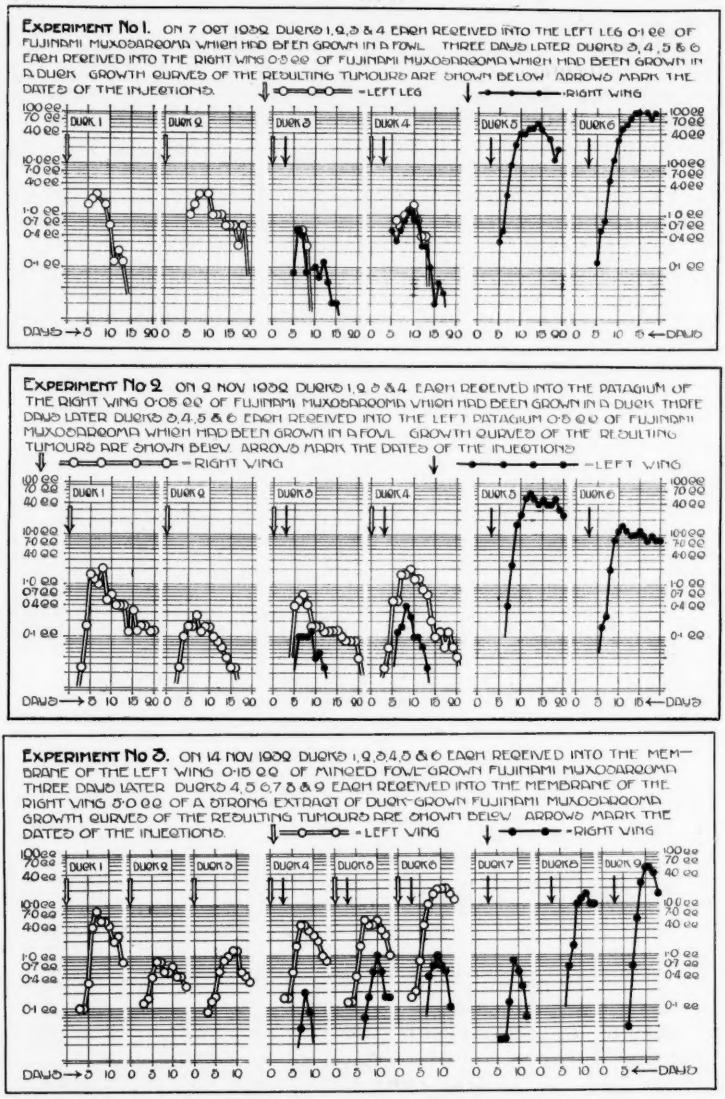
EXPERIMENT No 1. ON 7 OCT 1932 DUCKS 1,2,3 & 4 EACH RECEIVED INTO THE LEFT LEG 0·1 CC OF FUJINAMI MYXOSARCOMA WHICH HAD BEEN GROWN IN A FOWL. THREE DAYS LATER DUCKS 3,4,5 & 6 EACH RECEIVED INTO THE RIGHT WING 0·5 CC OF FUJINAMI MYXOSARCOMA WHICH HAD BEEN GROWN IN A DUCK. GROWTH CURVES OF THE RESULTING TUMOURS ARE SHOWN BELOW. ARROWS MARK THE DATES OF THE INJECTIONS.
= LEFT LEG
= RIGHT WING
DUCK 1
DUCK 2
DUCK 3
DUCK 4
DUCK 5
DUCK 6
DAYS
EXPERIMENT No 2. ON 2 NOV 1932 DUCKS 1,2,3 & 4 EACH RECEIVED INTO THE PATAGIUM OF THE RIGHT WING 0·05 CC OF FUJINAMI MYXOSARCOMA WHICH HAD BEEN GROWN IN A DUCK. THREE DAYS LATER DUCKS 3,4,5 & 6 EACH RECEIVED INTO THE LEFT PATAGIUM 0·5 CC OF FUJINAMI MYXOSARCOMA WHICH HAD BEEN GROWN IN A FOWL. GROWTH CURVES OF THE RESULTING TUMOURS ARE SHOWN BELOW. ARROWS MARK THE DATES OF THE INJECTIONS.
= RIGHT WING
= LEFT WING
DUCK 1
DUCK 2
DUCK 3
DUCK 4
DUCK 5
DUCK 6
DAYS
EXPERIMENT No 3. ON 14 NOV 1932 DUCKS 1,2,3,4,5 & 6 EACH RECEIVED INTO THE MEMBRANE OF THE LEFT WING 0·15 CC OF MINCED FOWL-GROWN FUJINAMI MYXOSARCOMA. THREE DAYS LATER DUCKS 4,5 & 7,8 & 9 EACH RECEIVED INTO THE MEMBRANE OF THE RIGHT WING 5·0 CC OF A STRONG EXTRACT OF DUCK-GROWN FUJINAMI MYXOSARCOMA. GROWTH CURVES OF THE RESULTING TUMOURS ARE SHOWN BELOW. ARROWS MARK THE DATES OF THE INJECTIONS.
= LEFT WING
= RIGHT WING
DUCK 1
DUCK 2
DUCK 3
DUCK 4
DUCK 5
DUCK 6
DUCK 7
DUCK 8
DUCK 9
DAYS

FIG. 1.—The results of all three experiments show that, in ducks, resistance to a second dose of Fujinami agent is well developed in so short a time as 3 days. In each experiment compare the effect of the second doses in the second group of ducks (black curves) with the effect of similar doses in normal birds (the third group of ducks).

grew much less freely than those developing in Ducks 5 and 6, although these latter received similar doses of the same material. This failure of the second inocula to produce as great a response in Ducks 3 and 4 as in normal ducks can be attributed only to changes initiated by the first injections. Experiments 2 and 3, as shown in Fig. 1, gave similar results ; after only three days a duck is incapable of responding in the same degree as a normal duck. The difference is too constant and too great to be attributable to other causes. In the experiments illustrated it is shown that the tumours produced in ducks previously inoculated may be only one tenth the mass of those produced in normal birds in response to similar inocula. In Experiment 3, Fig. 1, cell-free extract, which here was of unusually high concentration, was used for the second inoculations. Extract was used to avoid effects which might be attributable to living cells in this second inoculum. The precaution was hardly necessary, for it was already known (Purdy, 1933, *a*; 1933, *b*) that in adult ducks, even when minced tissue is inoculated, the new tumours are formed solely from infected host-cells and not at all from surviving cells of the inoculum.

It is difficult to say just how early inoculated ducks become totally refractory to a second inoculation. Mince of somewhat low tumour-producing power has completely failed to produce tumours when injected in 2·0 c.c. doses on the fourth day, whilst mince of higher potency has produced small tumours when given on the fifth day. Thus the refractory state becomes fully established at about the same time as regressive changes appear in the tumour by which the refractory state is induced. Whether the power of resisting re-infection and the power of overcoming an established infection are both simply different aspects of the same phenomenon cannot be stated. No experiment yet devised has proved adequate to the solution of this problem. In the fowl there is practically no tendency to spontaneous cure of the Fujinami tumour, and no increased resistance to re-infection has been detected in experiments analogous to those illustrated in Fig. 1. But here again it is open to question whether the two facts are closely related.

No evidence has been found that the Rous sarcoma No. 1 growing in ducklings renders the host refractory to the Fujinami infection. When tests have been made on a sufficiently large scale the results have always been the same. One such experiment is summarized in Fig. 2. Newly hatched ducklings were used. The experiment was carried out on lines similar to those adopted for the experiments summarized in Fig. 1 and already described. Study of the growth curves reproduced in Fig. 2 shows that the tumours induced by the extract of Fujinami myxosarcoma were not materially larger in Ducklings 6, 7, 8, 9 and 10 than in Ducklings 1, 2, 3, 4 and 5, although the birds of the latter group had previously borne Rous sarcomas. Duckling 2 was the only anomalous bird. In it the extract of Fujinami myxosarcoma produced no tumour. The reason for this failure is not understood. Similarly anomalous birds have been met with in other experiments of this kind. The result of the experiment taken as a whole is regarded as evidence that in ducklings a Rous sarcoma does not protect against a subsequent injection of potent extract of Fujinami myxosarcoma. It is not known whether, in this respect, it is significant that the Rous sarcoma growing in ducklings consists of fowl-cells (Purdy, 1933, *a* ; 1933, *b*), and not of duck-cells.

In ducks it seems impossible to test whether a Rous sarcoma, as such, renders the host refractory to a subsequent inoculation of minced Rous tumour. For one thing, by the time the first Rous sarcoma has grown and begun to subside again the duckling is approaching an age at which it would become refractory in any case. There is also the fact that the first Rous sarcoma, consisting as it must of implanted cells—implanted cells of a foreign species of bird in fact—may be expected for that reason alone to induce resistance to

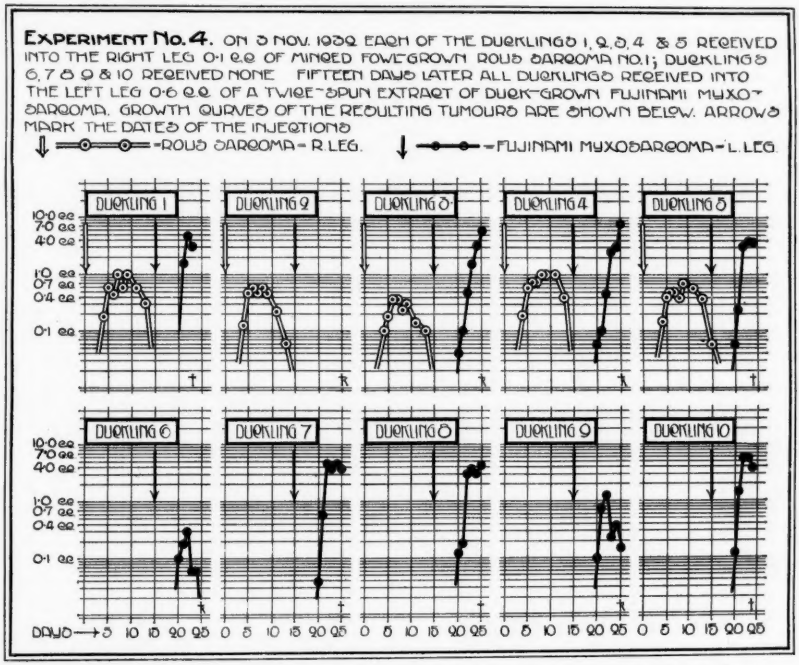
EXPERIMENT No. 4. ON 3 NOV. 1932 EACH OF THE DUCKLINGS 1, 2, 3, 4 & 5 RECEIVED INTO THE RIGHT LEG 0·1 C.C. OF MINCED FOWL-GROWN ROUS SARCOMA NO. 1; DUCKLINGS 6, 7, 8, 9 & 10 RECEIVED NONE. FIFTEEN DAYS LATER ALL DUCKLINGS RECEIVED INTO THE LEFT LEG 0·6 C.C. OF A TWICE-SPUN EXTRACT OF DUCK-GROWN FUJINAMI MYXO-SARCOMA. GROWTH CURVES OF THE RESULTING TUMOURS ARE SHOWN BELOW. ARROWS MARK THE DATES OF THE INJECTIONS.
= ROUS SARCOMA - R. LEG.
= FUJINAMI MYXOSARCOMA - L. LEG.
DUCKLING 1
DUCKLING 2
DUCKLING 3
DUCKLING 4
DUCKLING 5
DUCKLING 6
DUCKLING 7
DUCKLING 8
DUCKLING 9
DUCKLING 10
10·0 cc
7·0 cc
4·0 cc
1·0 cc
0·7 cc
0·4 cc
0·1 cc
DAYS → 5 10 15 20 25

FIG. 2.—In ducklings the growth of a Rous sarcoma No. 1 produces no resistance to infection by the Fujinami agent. Compare the black curves in the upper series of charts with those in the lower series.

any subsequent implantation of cells of the same species of animal, whether malignant or not.

No evidence has been found that a Rous sarcoma growing in fowls renders the host refractory to a subsequent inoculation. Perhaps the results would have been more significant had a slow-growing strain of Rous sarcoma been used in the experiments, but none was available. From what has been done, however, it is concluded that with a Rous strain of ordinary malignancy the refractory state, if it occurs at all, is not readily demonstrable in fowls by methods similar to those used in the experiments summarized in Fig. 1.

SUMMARY.

Normally, adult ducks recover from a Fujinami infection and are then solidly immune to the disease.

It has now been shown that this immunity to subsequent infection is well developed by the third day, and that immunity becomes complete within the following few days.

It has now been shown, too, that a Rous sarcoma No. 1 growing in a duckling does not induce immunity to a Fujinami infection.

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Idem AND PURDY, W. J.—(1931) 'The Cause of Cancer'. London (Cassell & Co.).
PURDY, W. J.—(1932) *Brit. J. Exp. Path.*, **13**, 467; (1933, *a*) *ibid.*, **14**, 9; (1933, *b*) *ibid.*, **14**, 260.

SPECIFIC AGGLUTINATION OF BACTERIOPHAGE PARTICLES.

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UNTIL very recently there has been no serious suggestion in bacteriophage literature that the phage-antiphage reaction can be observed by any technique other than the "phage-neutralization" method introduced by Bordet and Ciuca (1921), and modified by many other workers. With the realization that some phages at least were composed of considerably larger particles than was currently supposed (Elford and Andrewes, 1932; Schlesinger, 1932), the possibility of obtaining demonstrable agglutination by antiphage serum had to be considered. I have previously (1933, *b*) noted briefly that true macroscopic agglutination of a large particle phage (C16) can occur, and in a very recent paper Schlesinger (1933) describes specific agglutination of his phage "W.L.L." when observed ultramicroscopically. Through the kindness of Dr. Schlesinger I have been able to compare this phage with those in my possession. It is a typical example of serological Group 11 (Burnet, 1933, *a*), and in every respect is hardly distinguishable from C16. Both phages therefore possess particles of the largest size estimated by Elford as 50–75 mμ. in diameter by Schlesinger as 79–90 mμ. The present communication is concerned with the conditions under which macroscopic agglutination (or precipitation) of phages of this particular size and serological group occurs, and with the microscopic

appearance of the aggregates obtained. The results are not necessarily applicable to phages of any other group.

Technique.

The phage C16 has been fully described previously (Burnet, 1933, *a*). It is an active small plaque large particle phage, a typical example of what is probably the commonest form of dysentery-coli phage. The corresponding antiserum has also been described in the same paper. It is of unusually high titre showing detectable inactivating effect at dilutions as high as 1 : 200,000. The crude serum contains agglutinins for smooth and rough dysentery bacilli, and except where stated otherwise, all the experiments described have been made with serum from which these agglutinins have been absorbed with S and R strains of Y. After absorption the serum was filtered either through a Seitz disc or more usually through a relatively permeable gradocol membrane. This precaution was probably not strictly necessary as the crude serum gave no reaction whatever with any serologically unrelated phage, the bacterial precipitinogen in the lysed cultures being apparently not in sufficient concentration to react visibly with the serum agglutinins.

Washed phage preparations were kindly made for me by Dr. Elford. The lysed culture was first filtered through a permeable (about 0·8μ A.P.S.) membrane, and the filtrate washed over a membrane impermeable to the phage particles, *e. g.* of 50 mμ. A.P.S., by reducing the volume to about 1/10, then making up to the original volume with broth or Ringer solution and continuing the process for 5 or 6 repetitions.

Agglutination reactions were carried out aseptically in small plugged tubes kept in a water-bath at 50°. Some reaction was often visible after 3 or 4 hours, but the routine readings were made after the tubes had been left in the bath overnight.

1. THE NATURE OF THE AGGREGATION.

When a strong phage of titre round 1×10^{10} is treated with a suitable dilution of absorbed antiserum the aggregates produced after 24 hours are readily visible to the naked eye as a slight deposit. If the tube is gently shaken and examined with a lens, small compact floccules will be observed which resemble the aggregates of a weak O-type agglutination rather than the floccular product of most precipitin reactions.

The intensity of the reaction seems to depend almost entirely on the titre of phage suspension used. No reaction is given unless the original titre of the material used was 2×10^9 or higher, but in the range from this value to a maximum of 5×10^{10} the density of the precipitate allows a very fair estimate of the titre of a given preparation to be made. The concentration of antiserum, provided it is sufficient to cause aggregation, has only a slight influence on the bulk of the precipitate. The end-point of the reaction with falling serum dilutions is fairly sharp, and by taking a standard intensity of aggregation one can compare the titres of two serums with about the same accuracy as would be obtained with two bacterial agglutinating serums. A protocol (Table I) shows the intensity of aggregation in conventional symbols in relation

to varying phage and serum concentrations. To allow the use of stronger serum unabsorbed antiphage serum was used in this experiment, and the phage agglutinated was grown on a different strain (coli R) from that on which the phage used for immunization was developed (Y.S). The phage titre was almost exactly 10^{10} by plaque counts.

TABLE I.

Final serum dilutions.	Final dilutions of phage. 2.	4.	8.	20.	40.
4	+++	+±	±	−	−
20	+++	++	±	−	−
40	+++	++	−	−	−
80	++	++	−	−	−
160	+	±	−	−	−
320	−	−	−	−	−

+++ easily visible to naked eye. ++ just visible to naked eye.
Dilutions in Ringer solution incubated in water-bath at 50° overnight.

Such findings strongly suggest that the bulk of the precipitate is provided by the antigen not by the antiserum, *i. e.* the reaction is an agglutination rather than a specific precipitation.

It is immaterial in what form the phage particles are presented provided they are in sufficient concentration. Washed phage particles suspended either in broth or in Ringer give typical aggregations of precisely similar appearance to those obtained from crude filtrates of the same phage titre. Phage grown on *B. coli* in synthetic medium reacts well; the heaviest precipitate I have seen was obtained with one of Schlesinger's lightly turbid phage filtrates (Schlesinger, 1933), grown on synthetic medium.

Phage inactivated by heat, 70° C.–85° C. for 30 minutes, is still agglutinable, but the agglutinability is lost if the phage is brought to 100° for 15 minutes. Formalin killed phage (0·25% formalin) shows undiminished reactivity as does phage inactivated by exposure to light in the presence of a suitable photodynamic dye. (C16 is relatively resistant to methylene blue-light inactivation, and the more active proflavine was used in this experiment.)

All the findings therefore indicate that the aggregation is a true agglutination dependent upon the antigenic structure of the surface of the phage particles and independent of whether they are alive or dead. This conclusion is strengthened by the results of microscopic examination of the precipitates.

2. THE RANGE OF PHAGES AGGLUTINATED.

Provided the filtrates available had a sufficiently high titre, all the phages falling into Group 11, *i.e.* in activated by an anti-C16-serum, were also agglutinated by this serum. None of the serologically unrelated phages showed any suggestion of agglutination by anti-C16-serum. The phages which have been tested are as follows:

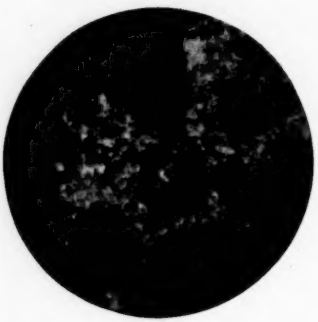

FIG. 1.

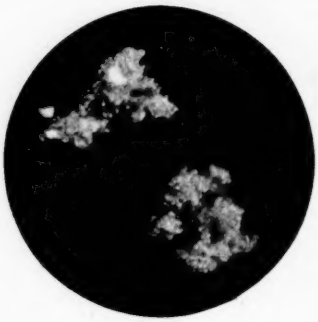

FIG. 2.

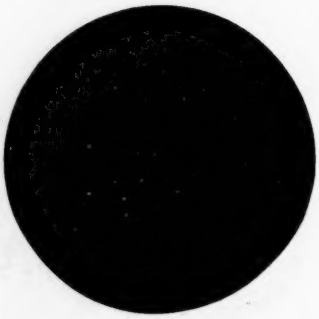

FIG. 3.

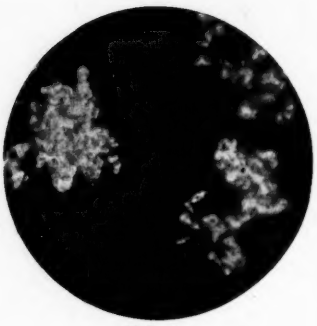

FIG. 4.

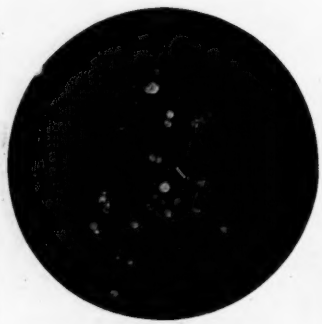

FIG. 5.

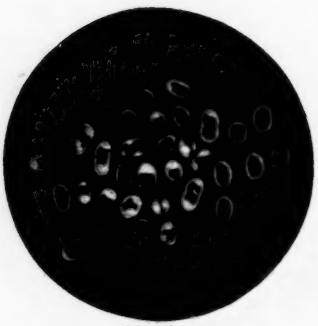

FIG. 6.

Agglutinated : C16, D12, D29, H, J, K, C4 : C20 ± (all in Group 11).
Not agglutinated : S13, C13, D44, D5, C36, D13, D3, C18, L, C21.

This limitation of agglutinability to the phages which can be inactivated by the serum makes it highly probable that only one antibody is concerned in the two reactions.

3. IDENTITY OF AGGLUTINATING AND PHAGE-INACTIVATING ANTIBODIES.

It is a notoriously difficult immunological problem to decide rigidly whether serological reactions involving one antigen, but demonstrated by quite different techniques are or are not due to the one type of antibody. The present attitude may be fairly stated as inclined to assume that only one type of antibody is involved, unless very cogent evidence for the necessity of invoking more than one can be produced. It has already been shown (Burnet, 1933, *c*) that the *phage inactivating* antibody can be absorbed from a serum by means of phage-coated bacteria, and that simultaneously with the reduction in this antibody activity there is a loss of the power to agglutinate the phage-coated bacteria. The phage inactivating power of the serum is therefore due to something which can be adsorbed on to the surface of the phage particles, and it is logical to assume that the same adsorption of antibody is responsible for the changes in suspension stability shown by both the specifically-coated bacteria and the phage particles themselves. Evidence to the contrary has been sought in two directions. A globulin preparation was made from high titre antiphage serum and its agglutinating and phage-inactivating activity compared with the original.

Similarly the two activities were estimated for the serum of an immune rabbit before and after the secondary antigenic response to an injection of phage C16. As will be seen from the accompanying protocol (Table II) in each instance there is an approximately constant relationship between the phage inactivating and agglutinating titres, the former being about 1000 times the latter under the conditions used.

The globulin preparation was kindly made by Dr. G. A. C. Gough, using 1/2 saturation with ammonium sulphate, dialysis and final solution in the original volume of normal saline. In order to obtain a sterile preparation this was filtered through a gradocol membrane, an equal volume of broth being added to minimize loss during filtration.

EXPLANATION OF FIGURES.

All photographs taken with monochromatic ultra-violet light (× 3200).

FIG. 1.—Phage C16 (crude filtrate) agglutinated by the homologous antiserum.

FIG. 2.—Washed phage particles agglutinated by antiphage serum.

FIG. 3.—Unagglutinated particles in a concentrated phage filtrate prepared by Schlesinger's technique on synthetic medium, and further concentrated over an impermeable gradocol membrane. This preparation was highly opalescent.

FIG. 4.—Precipitate produced with Shiga polysaccharide at limiting dilution (1 : 10,000,000) and homologous antiserum to show the absence of the granular structure characteristic of agglutinated phage.

FIG. 5.—Canary-pox virus particles.

FIG. 6.—*B. prodigiosus.*

TABLE II.—*Relative Phage Inactivating and Agglutinating Titres of Different Sera.*

	Phage-inactivating titre.	Phage-agglutinating titre.
Original serum R4 . . .	60,000	80
Globulin solution from R4 .	10,000	10–20
Serum R3 before reinjection .	11,000	8–16
Serum R3 8 days after reinjection with C16	80,000	80

The phage-inactivating titres were determined by the method previously described (Burnet, 1933*c*) and represent the serum dilution capable of reducing the plaque-count to 20% under standard conditions. Agglutinating titres were fixed by taking one tube (the 1 : 80 serum dilution of the stronger preparation in each case) as an arbitrary standard and comparing the intensities of agglutination in the other series with this.

4. MICROSCOPIC STRUCTURE OF AGGREGATES.

Under high-power dark-ground illumination the aggregates appear to be of granular structure. This is confirmed by the microphotographs taken with ultra-violet light by Mr. J. E. Barnard. These are reproduced in the accompanying plate, and show that the masses are almost entirely composed of refractile granules which appear to be just below the size necessary for clear resolution by this technique. For comparison a microphotograph is shown of the floccules produced by the interaction of a very dilute specific bacterial carbohydrate solution and the homologous antiserum. A highly purified preparation of Shiga polysaccharide, kindly given to me by Dr. W. T. J. Morgan, was used at its limiting dilution, 1 : 10,000,000, with the homologous serum diluted 1 : 10. The slow precipitation induced gave a product whose microscopic structure was much more finely granular than those obtained more rapidly with stronger antigen solutions. When the microphotographs of this precipitate are compared with those of the phage aggregates, it is seen that the phage preparation is of more uniform granular texture with semi-isolated particles of characteristic size visible at the edge of the masses. The polysaccharide-antiserum precipitate contains granules of varying size many much larger than the phage ones imbedded in material which appears completely amorphous.

The photographs in themselves do not convey a great deal of information, and it is conceivable that under certain conditions specific precipitates from soluble reagents might produce aggregates of similar microscopic appearance. But on the other hand, if virus-like particles of the size found for phage C16 by filtration methods 50–75 mμ. were aggregated into masses by specific serum agglutination, then one would expect that the image obtainable with the available technique would be exactly similar to what is in fact found. It is not possible to give any accurate estimate of the diameter of the phage particles from these photographs, but they indicate that it is probably not much more than 50 mμ.

DISCUSSION.

The results reported in this paper add a further confirmation to the view that phages of the type of C16 (serological Group 11 of a previous paper) are distinctive living organisms whose size is just at the limits of modern microscopic technique. Since the coli phage used by Schlesinger is serologically and functionally almost identical with C16—and two of the same group D4 and D12 were studied by Elford and Andrewes in their filtration studies—a wide range of data in regard to the physical, functional and serological characters of phages of this group is now available. In regard to size Elford and Andrewes (1932) from filtration studies with gradocol membranes place the particle diameter at 50–75 mμ., Schlesinger (1932) using a centrifuge technique gives a somewhat higher estimate, 79–90 mμ., while the character of the image obtained with ultra-violet light microphotographs of agglutinated phage particles suggests a value around 50–60 mμ. There can, therefore, be no question that the particles are at least 50 mμ. in diameter and further the filtration, centrifuge and microphotographic results all indicate that the particles concerned are of approximately uniform size.

The experimental data given seem to be sufficient to show that the precipitate obtained when high titre phage and antiphage serum are mixed can only represent agglutinated phage particles. The only other conceivable interpretation that the phage particles are mechanically entangled in the precipitate produced by the interaction of soluble phage-specific antigen and antibody is eliminated by the use of washed phage suspensions. It is convenient to summarize here the various methods, described in the present and two previous papers (Burnet, 1933, *c* and *d*), by which phage-antiphage reactions may be demonstrated using phage C16 or other phages of the same serological group. These are :

(*a*) By the classical method of inhibition of lytic activity.

(*b*) By the use of phage-coated bacteria which

i. Are agglutinated by the specific antiphage serum, and

ii. Can absorb the phage inhibiting activity from such serum.

(*c*) By agglutination of phage particles, either living or killed by certain methods.

(*d*) The existence of phage-specific soluble substances can be shown by

i. The blocking effect on phage-antiphage reaction (*a*), or

ii. If in high concentration by the appearance of a specific precipitate.

Although direct evidence on the point is incomplete, it is simplest to regard all the reactions as being manifestations of a single antibody corresponding to the specific antigen of the bacteriophage surface. It will be obvious that these results are closely analogous to many from the domain of bacterial immunology, and it is suggestive that fundamentally similar series of antigen-antibody reactions have now been described for the pneumococcus, for vaccinia virus (Craigie, 1932 ; Wilson Smith, 1932) and for a bacteriophage.

CONCLUSIONS.

One of the large particle phages (C16) can be specifically aggregated by homologous high titre antiphage serum.

The aggregates when examined by ultra-violet light microphotography appear to be composed of granules whose optical properties suggest that they are of approximately uniform size and about 50 mμ. in diameter.

The reaction can be obtained with washed phage preparations and sera freed of bacterial agglutinins and must represent a true agglutination of the phage particles.

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DETOXICATION OF SNAKE VENOMS, AND THE APPLICATION OF THE RESULTING ANTIGENS TO RAPID METHODS OF ANTIVENOMOUS VACCINATION AND SERUM PRODUCTION.

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THE object of this work will appeal more particularly to those who have experienced the difficulties inseparable from the regular production of anti-venom serum on a large scale. We set out to obtain a practical method of immunization by which large doses of atoxic venom derivatives might be administered over a short period of time, giving rise to a polyvalent anti-venom of high potency, and thus overcoming the hazardous and protracted methods of production based on the classical work of Calmette, to whom we owe the existence of a specific antivenomous serum therapy.

DETOXICATION OF VIPERINE AND COLUBRINE VENOMS BY MEANS OF VARIOUS ORGANIC AND INORGANIC SUBSTANCES.

Calmette, Phisalix and Bertrand among others have investigated the effect of heat, whilst various chemical aspects have been studied by Calmette

(1894), Flexner and Noguchi (1904), Ramon (1925), Bächer, Kraus and Loewenstein (1925), Martin and Lamb (1907), Martin (1925), Arthus (1930), Vital Brazil and Vellard (1927), and Vellard (1930). With the exception of the results obtained by the last two workers, who evolved a method of preparing *viperine* anti-venom serum, utilizing lipoidal extracts of hepatic and other origins, the remainder are of interest on general principles rather than from a practical point of view.

Of the large number of substances employed in the course of our detoxication experiments, we shall deal only with ox bile, organic gold salts (solganal) and formalin; since these alone held out fair prospects of practical success and utility, they were the products which formed the basis of our work.

The multiplicity of the species of snakes to be found throughout south and equatorial Africa demands a highly polyvalent anti-venom serum, so that in our search for a suitable, practical detoxicating agent, its effect on both viperine and colubrine venoms was an important criterion. Moreover, from the outset we realized the fundamental importance of utilizing strong concentrations of venom, the small practical value of the studies hitherto carried out by various workers being, in our opinion, largely due to the weak solutions of the venom employed; neither viperine nor colubrine venoms were employed by us in solutions weaker than 1 per cent., whilst a considerable amount of work was performed on 2, 4 and even 10 per cent. solutions. Large quantities of atoxic venom derivatives could thus be administered in small volumes—an important practical consideration, both in the hyper-immunization of large animals for serum production and in active anti-venomous vaccination of domestic animals, monkeys, etc.

Detoxication of Venoms by Means of Bile.

Bile seems always to have been credited with powers of neutralizing venoms and, in fact, still remains a popular if ineffective method of treating cases of snake-bite. Sterilized ox bile was one of the first agents employed by us, parallel experiments being undertaken on both viperine and colubrine venoms. One per cent. solutions of fresh, recently desiccated *Bitis arietans* (Puff adder), *Causus rhombeatus* (Night adder), and *Naia flava* (Cape cobra) venoms were employed, these being incubated at 37° C. in the presence of from 1 per cent. to 5 per cent. sterilized ox bile, the toxicity being tested at weekly intervals; the test flasks were always accompanied by controls consisting of 1 per cent. solutions of venom in normal saline.

Bitis arietans (Puff adder) Venom.

The fatal dose of desiccated *Bitis arietans* venom employed for control purposes was 2 mgm., this quantity when administered intravenously killing 2000-gm. rabbits in 2½ hours.

As a result of the action of 5 per cent. ox bile at 37° C., 1 per cent. solutions of *Bitis arietans* venom were rendered atoxic in 28 days, test rabbits successfully resisting the equivalent of ten fatal doses intravenously. After even 42 days, however, neither 1 per cent. nor 2·5 per cent. bile was able to detoxicate completely, test rabbits dying regularly in from 1 hour upwards, according to the test dose employed.

Causus rhombeatus (*Night adder*) *Venom.*

At least one month's incubation in the presence of 5 per cent. ox bile was necessary for detoxication ; smaller proportions of bile exerted a somewhat incomplete effect, as shown by tests carried out at the end of even three months' incubation.

Naia flava (*Cape cobra*) *Venom.*

Guinea-pigs were here employed as the test animals, a subcutaneous injection of 0·6 mgm. being a certainly fatal dose in 2½ hours. Five per cent. bile appeared to exert no appreciable detoxicating action, at any rate on the 1 per cent. solutions employed by us, for animals tested with material which had been incubated for nine months died with negligible delay as compared with controls.

A number of interesting experiments were carried out on the detoxicating action of reptilian bile ; snakes and tortoises were employed for the purpose, and it was found that *Bitis arietans* venom was readily rendered atoxic as the result of the action of *Bitis arietans* bile at incubator temperature.

As far as our work is concerned, ox bile possesses the advantages of being more easily obtainable, and exerting a much more rapid action than the lipoidal extracts elaborated by Vellard in South America, and recently employed in India on Asiatic viperine venoms by Maitra and Mallick (1932).

Detoxication of Venoms by Means of Solganal.

The detoxicating action of this organic gold preparation on diphtheria and tetanus toxin has been studied by one of us (E. Grasset). The results obtained, which are in course of publication, held out encouraging prospects where venoms are concerned. Experiments showed that, in contrast to the rapidly destructive action of the inorganic gold preparations noted by Calmette and others, the organic product exerted the same gradual action on viperine venom as was found to be the case in the transformation of diphtheria and tetanus into antigenic products analogous to anatoxins ; it exerted, however, little if any effect on *Naia flava* venom.

On allowing 1 per cent., 0·5 per cent. and 0·25 per cent. concentrations of solganal to act on 1 per cent. solutions of venom in normal saline, *Bitis arietans* venom was rendered practically atoxic by one month's incubation at 37° C. ; under the same conditions *Naia flava* venom was not appreciably affected.

Detoxication by Means of Formalin.

Varying quantities of formalin were allowed to act for varying periods on a large number of venoms, including *Bitis arietans*, *Bitis cornutus*, *Causus rhombeatus*, *Naia flava*, *Naia haie*, *Naia nigricollis* and *Dendraspis angusticeps*.

Table I summarizes the detoxicating action of formalin on 1 per cent. solutions of venom in normal saline.

Having thus succeeded in obtaining atoxic venom derivatives, immunization experiments were undertaken. It was soon evident, however, that whereas

TABLE I.—*Detoxicating Action of Formalin on 1 per cent. Solutions of Venom in Normal Saline.*

Venom.	Formalin (%).	Temperature (°C.).	Test animals.	Result.
Bitis arietans	1	37	Rabbits	Atoxic after 28 days.
,, ,,	0·75	37	,,	,, ,, 28 ,,
,, ,,	0·5	37	,,	,, ,, 28 ,,
,, ,,	0·25	37	,,	,, ,, 42 ,,
,, ,,	1	56	,,	,, ,, 21 ,,
,, *cornutus*	1	37	,,	,, ,, 14 ,,
Causus rhombeatus	1	37	,,	Animals die immediately.
,, ,,	1	37	Pigeons	,, ,, ,,
Naia flava	1	37	Guinea-pigs	Not atoxic even after four months.
,, ,,	1	56	,,	Atoxic after 28 days.
Sepedon hæmachates	0·75	37	Rabbits	No appreciable detoxicating action.

Rabbits and pigeons were tested intravenously and guinea-pigs subcutaneously.

the viperine derivatives were well tolerated by animals, the colubrine derivatives produced considerable reactions which interfered with the course of the immunization; these reactions were considered as being partly due to the heavy, coarse precipitate resulting from incubation at the relatively high temperature of 56° C., and largely due to residual formalin. Reactions of this nature are not observed in immunization with diphtheria or tetanus anatoxins prepared by the action of formalin on broth filtrates, so that it seemed logical to dissolve *Naia* venom in broth instead of saline in the hope of not only reducing the ultimate quantity of uncombined formalin, but of deriving some immunological benefit from the presence of aspecific protein. This proved to be the successful turning-point in the detoxication of colubrine venoms, for whereas 1 per cent. *Naia flava* venom in saline was not detoxicated by a period of incubation extending over months, when Martin's broth was used instead of saline, as little as 0·75 per cent. formalin was successful in detoxicating this venom in slightly over two weeks' incubation at 37° C.

TABLE II.—*Detoxicating Action of Formalin on 1 per cent. Solutions of Venom in Martin's Broth.*

Venom.	Formalin (%).	Test animals.	Result.
Naia flava	1	Guinea-pigs	Atoxic after 21 days.
,, ,,	0·75	,,	Atoxic in from 21 to 28 days.
,, ,,	0·5	,,	Still toxic after some months.
Sepedon hæmachates	1	,,	Atoxic after 14 days.
,, ,,	0·75	,,	,, ,, 21 ,,
,, ,,	0·5	,,	Still toxic after some months.
Naia nigricollis	1	,,	Atoxic after 21 days.
,, *haie*	1	,,	,, ,, 21 ,,
Dendraspis angusticeps	1	,,	,, ,, 21 ,,
Bitis arietans	0·5	Rabbits	No appreciable detoxicating action.
,, *cornutus*	1	,,	Atoxic after 14 days.
Causus rhombeatus	1	Pigeons	,, ,, 7 ,,

Both 2 per cent. and 4 per cent. solutions of *Naia flava* venom were rendered atoxic by 1 per cent. formalin when incubated at 37° C. for 42 days.

Table II summarizes the results obtained by substituting Martin's broth for saline ; 1 per cent. solutions were employed in every case, the temperature being 37° C. Rabbits and pigeons were tested intravenously, and guinea-pigs subcutaneously.

From the large number of animals and birds experimented upon, the interesting and important fact emerges that whilst Martin's broth makes possible the ready detoxication of *Naia*, *Sepedon*, *Dendraspis* and *Causus* venoms, it inhibits the detoxication of *Bitis arietans* venom. In this respect the venom of the viper *Causus rhombeatus* appears to be more akin to the colubrine venoms than to that of the typical viper *Bitis arietans* ; as a general rule, however, all the colubrine venoms can be rapidly detoxicated in broth, but only very slowly in saline, at any rate in the concentrations of 1 per cent. employed by us. In order to ascertain which fraction of the broth was responsible for the detoxicating action, *Naia flava* venom was dissolved in both the veal infusion and the peptone solution which together comprise Martin's broth ; the peptone solution exerted a very slight detoxicating action, 1 per cent. solutions of venom being almost atoxic after three months' incubation in the presence of 1 per cent. formalin ; the veal infusion-venom solutions, however, were still highly toxic at the end of this period. Neither the veal infusion nor the peptone solution therefore assists to any extent the detoxication, in marked contrast to the action displayed by the two combined in the form of Martin's broth.

DETERMINATION OF THE COMPARATIVE ANTIGENIC PROPERTIES OF THE PRODUCTS RESULTING FROM DETOXICATION.

Having succeeded in detoxicating yiperine and colubrine venoms on a practical scale, we proceeded to ascertain the antigenic properties of the derivatives so obtained. The technique consisted of a series of injections and reinjections, the nature and degree of the immunological response being followed up step by step.

Immunization with Atoxic Bile-venom Derivatives.

The antigen consisted of 1 per cent. solutions of *Bitis arietans* venom in saline incubated at 37° C. for one month in the presence of 5 per cent. sterilized ox bile. Rabbits which received two subcutaneous injections, together totalling 200 mgm., were, ten days after the last injection, able to withstand successfully an intravenous injection of 1 cgm. fresh and newly desiccated *Bitis arietans* venom ; this test dose killed control rabbits in ten minutes. Analogous results were obtained on all the animals immunized, the high antigen properties of bile-treated *Bitis* venom being thus plainly evidenced. Nevertheless, the local reactions produced in guinea-pigs, rabbits and horses did not encourage a continuation of this method in view of the better results obtained with formalin.

Attempts at Immunization with Solganal-venom Derivatives.

As far as our investigations have proceeded up to the moment, solganal is mainly of interest in that, despite the fact that it detoxicates *Bitis* venom

TABLE III.—*Immunization Results with* 1 *per cent.* Bitis arietans *venom Incubated at* 37° *C. in the Presence of Formalin.*

Animal.	Formalin (%).	Number of injections.	Total antigen in terms of desiccated venom (mgm.).	Test in terms of desiccated venom (mgm.).	Result.	Control.
Rabbit	0·25	1	50	6	Survived	Died in 20 minutes.
,,	1	2	100	10	,,	,, 10 ,,
,,	1	3	120	15	Died, 4 hrs.	,, 8 ,,
*,,	0·25	3	250	20	Survived	,, 5 ,,
,,	0·25	3	150	15	,,	,, 8 ,,

* This rabbit was bled before being tested with venom; 1 c.c. of its serum was able to neutralize 2 mgm. desiccated *Bitis arietans* venom.

TABLE IV.—*Immunization Results with* 1 *per cent. Solutions of* Naia flava *venom in Martin's Broth Incubated in the Presence of* 1 *per cent. Formalin.*

Animal.	Number of injections.	Total antigen in terms of desiccated venom (mgm.).	Test in terms of desiccated venom (mgm.).	Result.	Control.
Rabbit	1	50	1·5	Survived	Died in 10 minutes.
Guinea-pig	1	20	3	Died, 4 hrs.	,, 45 ,,
Rabbit	2	100	2	Survived	,, 8 ,,
Guinea-pig	2	40	3	Died, 5 hrs.	,, 45 ,,
Rabbit	3	150	4	Survived	,, 6 ,,
Guinea-pig	3	60	5	,,	,, 40 ,,
Rabbit	4	325	15	,,	Died almost immediately.
,,	4	325	25	,,	,, ,, ,,

satisfactorily, experimental animals fail to stand a course of immunization with the atoxic derivatives; they sometimes die in a way that at present is not understood, but which seems to be unrelated to envenomation.

Immunization with Formalin-venom Derivatives.

Tables III and IV summarize the information afforded by some hundreds of estimations on pigeons, guinea-pigs and rabbits as regards their general and humoral immunity at progressive stages of immunization. Fresh desiccated venoms were employed for test and control purposes, rabbits being injected intravenously and guinea-pigs subcutaneously.

Formalin has thus proved to be satisfactory in the case of the concentrated solutions of venoms which we consider essential for practical results, and we now use it for detoxicating every batch of venom intended for large-scale immunization.

In contrast to the selective action displayed by bile and similar detoxicants, the wide range of action of formalin constitutes one of its chief advantages, and provided that broth or saline is appropriately chosen, every venom we have encountered may be readily detoxicated in one month or less without any appreciable loss of antigenic properties. The atoxic derivatives retain their antigenic stability, and the preservative action of formalin is of advantage in ensuring sterility. Very large doses, four or more grammes in the case of horses, may be safely administered in small volumes at intervals of a few days without undue local protein reactions, and with no evidence of envenomation.

The addition of tapioca to the antigen resulted in a stimulation similar to that observed in diphtheria and tetanus immunization.

THE EVOLVEMENT OF A PRACTICAL METHOD OF ACTIVE VACCINATION OF LABORATORY AND DOMESTIC ANIMALS.

Cases of snake-bite among animals are comparatively common in South Africa, and it was hoped to extend the principle of active vaccination to large domestic animals. The concentrated atoxic solutions employed were here of particular value, since the animals were able to tolerate massive doses of antigen irrespective of their natural sensitivity to snake venoms.

The animals chosen were sheep, goats, dogs, donkeys, mules, horses and monkeys.

Of the large number of animals employed, the following typical examples will serve to illustrate the technique and the nature of the response.

*Active Vaccination of Goats with Formalin-broth-*Naia flava *Derivatives.*

A goat, weighing 45 kg., received 0·5 gm. of antigen in the course of three subcutaneous injections, the interval between each being twelve days. The animal was tested with a subcutaneous injection of 15 mgm. desiccated *Naia flava* venom, and since no ill-effects were observed, a further 5 mgm. were administered, even this extra dose with its cumulative effect producing no obvious reaction.

Another goat weighing 50 kg. received a total of 3·1 gm. of antigen spread over five injections at intervals of a week. On being tested it was able to resist more than 40 mgm. *Naia flava* venom, and intravenous tests on rabbits proved that 1 c.c. of the goat's serum neutralized 0·4 mgm. of venom.

The results quoted are particularly noteworthy when it is remembered that goats and sheep are extremely sensitive to *Naia flava* venom, 2 mgm. being a certainly fatal dose in less than 12 hours. The above goats were therefore able to resist more than ten and twenty fatal doses respectively.

Active Vaccination of Goats and Sheep with Formalin-Saline-Bitis arietans *Derivatives.*

Goats which received 0·3 gm. of antigen in the course of three injections at intervals of a week were able to resist up to 200 mgm. desiccated *Bitis arietans* venom subcutaneously, this being equivalent to at least ten fatal doses.

Sheep vaccinated with either atoxic viperine or colubrine antigens afforded comparable results, whilst an equally high and rapid polyvalent immunity was provided by a number of goats and sheep which received *Bitis* and *Naia* antigens simultaneously.

Vaccination of Mules and Donkeys.

Polyvalent vaccinations of mules and donkeys served to illustrate the variation in response according to the species; the immunity conferred is comparable with that afforded by animals such as sheep and goats, but lower than that observed in the horse, whilst, as a general rule, mules appear to respond better and more rapidly than donkeys, their hybrid nature being a possible explanation.

Vaccination of Dogs and Monkeys.

A dog received 1·5 gm. of *Naia flava* antigen spread over four injections; three weeks after the last injection, 1 c.c. of its serum neutralized 0·5 mgm. of venom, whilst two weeks later the dog withstood a subcutaneous injection of 30 mgm. venom.

A dog which received 0·3 gm. of *Bitis* antigen in the course of four injections, was subsequently able to resist an injection of 75 mgm. desiccated venom.

A monkey weighing 5·5 kg. received 0·5 gm. of *Bitis* venom derivative over a series of four injections, and was subsequently able to resist a subcutaneous injection of 50 mgm. of venom. Twenty mgm. killed a control monkey in six hours. It will thus be seen that as far as atoxic viperine venoms are concerned, monkeys show a high response after a comparatively small immunization. Parallel vaccinations carried out with *Naia* antigen reveal a much lower response, and to obtain a satisfactory immunity one must resort to what almost amounts to a hyperimmunization.

THE HYPER-IMMUNIZATION OF HORSES.

The first portion of our work having enabled us to produce large quantities of atoxic antigens, we proceeded to study the response in horses; the animals

were divided into three groups—monovalent viperine, monovalent colubrine, and polyvalent.

One cgm. of *Bitis arietans* venom and 1·5 mgm. of *Naia flava* venom were the test doses employed for titrating the neutralizing properties of the sera, these doses killing rabbits in ten minutes when administered intravenously. The time of contact, at laboratory temperature, of mixtures of these test doses of venom and varying quantities of serum was usually one hour, although in order to study the subject of avidity the period was shortened in many cases. The titre of a serum was assessed as the weight of desiccated venom neutralized by a volume of 1 c.c.

The following typical examples will serve to illustrate the nature of the average response.

Monovalent Viperine.

Horse 126 received 1·6 gm. of atoxic *Bitis arietans* venom during the course of four subcutaneous weekly injections. Neutralization tests carried out five weeks after the commencement of the immunization showed that 1 c.c. of serum neutralized 7 mgm. of *Bitis arietans* venom. The animal tolerated the immunization without ill-effects and has, in fact, continued to furnish antivenine of high potency for more than a year with the same frequency of immunizations and bleedings as in the case of horses producing other antitoxins ; a resting period of three weeks is ample, after which a series of three injections employing a total of 6 gm. of antigen gives rise to a serum of even greater potency than before.

Monovalent Colubrine.

Horse 123 received 4·5 gm. of *Naia flava* venom derivative during a course of six injections. Seven weeks after the commencement of the immunization 1 c.c. of serum neutralized as much as 1·2 mgm. desiccated venom.

Polyvalent.

Horses 127 and 128 underwent a parallel course of immunization, each receiving a mixture of 4·98 gm. *Bitis* and 5·65 gm. *Naia* venom derivative during a course of ten injections. The animals were ready for bulk bleedings in less than three months from the commencement of the immunization, 1 c.c. of the serum of horse 127 neutralizing either 6·5 mgm. *Bitis* venom or 0·7 mgm. *Naia* venom, whilst 1 c.c. of the serum of horse 128 neutralized either 6 mgm. *Bitis* venom or 0·8 mgm. *Naia* venom.

As our studies progressed we were able to achieve still better results with a smaller total quantity of antigen by reducing the number of injections and increasing the quantity of antigen per injection. For example, in contrast to horses 127 and 128, which each received 10·63 gm. over a series of ten injections, may be cited horse 124, which received only six injections, utilizing in all 6·6 gm. of the antigen ; seven weeks after the commencement of the immunization, 1 of c.c. serum neutralized either 6·0 mgm. *Bitis* venom or 1·2 mgm. *Naia* venom.

The practical advantages of being able to prepare potent monovalent and polyvalent antivenenes in a few weeks can best be appreciated by considering the usual technique utilizing unmodified venoms; by commencing with minute doses which can only be gradually and hazardously increased, one is fortunate to obtain a serum of but moderate potency after a period of sixteen to eighteen months. Serious local and general reactions are inseparable from the process, the death of an animal during the later stages of the immunization being in the nature of a calamity.

Secure in being able to produce large quantities of high-titred antivenene with safety and regularity, we were in a position to consider the concentration of the product on lines analogous to those employed in the case of other antitoxins. The investigations have formed the subject of a number of publications (E. Grasset, 1932), and as a result, the antivenene is now regularly concentrated and refined so that 1 c.c. neutralizes either 10 mgm. *Bitis* venom or 1·8 mgm. *Naia* venom. Such sera are three times more potent than those usually produced by the classical method.

SUMMARY.

The action of a number of possible detoxicants of venoms has been studied. Concentrated solutions of both viperine and colubrine venoms are most readily detoxicated at 37° C. by means of formalin.

In general, viperine venoms are detoxicated more readily and more satisfactorily when dissolved in saline and colubrine venoms when dissolved in broth.

The atoxic derivatives are highly antigenic.

Domestic animals and monkeys have been actively vaccinated by a course of three or four subcutaneous injections.

By the hyper-immunization of horses, monovalent and polyvalent antivenenes of exceptional potency are obtained in a few weeks with ease and safety.

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THE INACTIVATION OF INSULIN BY NORMAL AND DIABETIC BLOOD.

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THE straightforward view that diabetes mellitus is due to a failure of the islet cells to secrete insulin is not wholly satisfactory. It does not explain the absence of correlation between diabetic symptoms and pathological changes in the pancreas, nor the occurrence of cases of diabetes (especially with infection) resistant to insulin treatment, nor the beneficial effect of diets high in carbohydrate and low in fat. Hence many extended or alternative theories of the ætiology of diabetes have been put forward, one of the most plausible being the suggestion that in diabetes insulin is destroyed in the blood-stream. Epstein and Rosenthal (1924) showed that the action of insulin was abolished by the simultaneous injection of trypsin, and Buckley (1931, 1933) found that rabbits which had received trypsin subcutaneously were resistant to insulin for several days thereafter. The theory that quantities of trypsin sufficient to destroy circulating insulin may at times be present in the blood can be used to explain the effect of various diets upon diabetes (Rabinowitch, 1932). Mauriac and Aubertin (1928) found that diabetic plasma neutralized insulin *in vitro* to a somewhat greater extent than normal plasma, and ascribed this to more complete escape of the neutralizing substance from the corpuscles. Karelitz, Cohen and Leader (1930) confirmed these findings, and obtained evidence that the neutralizing substance was an enzyme, possibly trypsin, inactive in acid solution. Watson and Dick (1933) believe that such an enzyme is present in urine, and that the amounts excreted are slightly increased in diabetes. The experiments to be described confirm the presence of an inactivating substance in blood, but they lend no support to the view that the amount of this substance is increased in diabetes.

INACTIVATION OF INSULIN BY SERUM.

Samples of diabetic blood were obtained from patients at the Montreal General Hospital, while non-diabetic patients furnished the normal bloods; none of the donors were suffering from infections. 10 c.c. samples of serum were incubated with 1 c.c. of normal saline containing 3 units of insulin for one hour at 37° C. 10 c.c. of the mixture was then injected subcutaneously into fasting rabbits, and the blood-sugar curves followed by the Shaffer-Hartman-Somogyi method. Each animal was used for tests with normal

and diabetic sera and for controls with saline in place of serum ; in this way the great variation between individuals was prevented from obscuring the results. That some inactivation of the insulin occurred was obvious ; convulsions occurred in 4 out of the 8 control experiments, and in none of those with serum-treated insulin. It was, however, also clear that there was no detectable difference between diabetic serum and normal serum. This was true both in the first series, in which the bloods were collected from fasting patients, and in the second and third series, in which the collections were made 1 or 1½ hours after a meal. The results of the first series (experiments with fasting serum) are presented in Table I. A group of 8 rabbits was used, each being tested twice with normal serum, twice with diabetic serum, and once with saline (plus 3 units insulin in each case).

TABLE I.—*Average Effect of the Subcutaneous Injection of 3 Units Insulin Incubated with Normal and Diabetic Sera.*

Serum.	Blood-sugar (mgm. per 100 c.c.).					
	Initial.	30 mins.	60 mins.	90 mins.	120 mins.	180 mins.
Normal	118	97	75	70	68	75
Diabetic	120	95	74	66	67	77
Control	114	79	58	47	46	45

INACTIVATION OF INSULIN BY LAKED CELLS.

1 c.c. of cells, obtained by centrifugation of citrated blood, was laked with 6 c.c. of water, and, in the presence of 3 c.c. of phosphate buffer (pH 7·35), incubated as before with 1 c.c. of saline containing a known amount of insulin, and the mixture injected subcutaneously. The apparent inactivation of the insulin was much greater than in the experiments with serum ; 3 units of insulin so treated had no effect upon the blood-sugar of the fasting rabbits. When larger quantities of insulin were used, it was found that the variations between individual animals became enormous ; thus rabbit 11 could be convulsed with 15 units of incubated insulin, while rabbit 24 resisted doses as large as 120 units. This variation made the averaging of results impossible, while it was also found inconvenient to make repeated observations on any one animal owing to the formation of abscesses at the site of injection (a difficulty which did not arise with the serum-treated insulin). In three animals it was possible to define a convulsion level, and no indication that diabetic cells differed from normal cells could be discerned. Since these experiments were not very satisfactory, and especially since it appeared probable that the "inactivation" observed was largely ascribable to delayed absorption from the site of injection, it was resolved to carry out similar experiments with intravenous injections. Small desensitizing doses of laked cells were given between experiments to prevent anaphylactic reactions.

The intravenous injection of laked cells does not in itself affect the blood-sugar. But it was surprising to find that neither normal nor diabetic washed, laked cells appreciably reduced the action of 5·5 units of insulin when the incubated mixture was injected intravenously. This finding contrasted

strikingly with the results of subcutaneous injections. When smaller quantities of insulin (3 units) were used, definite evidence of partial inactivation was obtained; but again there was no sign that diabetic cells were more potent in this respect than normal cells; if any difference exists, it is in the reverse direction. Table II reports the effect of injecting 3 units of insulin incubated with 1 c.c. laked red cells for one hour at 37° C., pH 7·35, in a total volume of 10 c.c.; a group of 13 rabbits was used for these experiments.

TABLE II.—*Average Effect of the Intravenous Injection of 3 Units Insulin Incubated with Laked Normal or Diabetic Cells.*

Cells.	Blood-sugar (mgm. per 100 c.c.).			
	Initial.	60 mins.	120 mins.	180 mins.
Normal . .	114	99	116	117
Diabetic . .	114	85	102	114
Control . .	117	53	76	102

A number of experiments were carried out in the hope of obtaining information as to the nature of the process of inactivation. It was found that in the experiments described above the washing of the cells is not important, but laking is essential. Without hæmolysis no inactivation of insulin occurs, although various attempts were made to cause the inactivating substance to diffuse out of the intact cells.

The properties of the solution of cell-contents are not lost by Berkefeld filtration; but they disappear after heating to 60° C. for 30 minutes. If the incubation is carried out at pH 6 no inactivation of insulin occurs; but adjusting the pH to this level after incubation at pH 7·35 does not restore the potency of the inactivated insulin. Crystallized hæmoglobin, freshly prepared from rat or dog blood, did not inactivate insulin in two hours' incubation at pH 7·6. These findings are in accord with those of previous investigators (Mauriac and Aubertin, 1928; Karelitz, Cohen and Leader, 1930). They suggest that the inactivation is due to the action of a proteolytic ferment; but the presence of the substance in the cells makes it improbable that trypsin of pancreatic origin is responsible, and it was possible to show that a potent preparation of antitrypsin, prepared according to the method used by Harned and Nash (1932) from the round-worm *Ascaris suillius*, protected insulin from the action of pancreatin (U.S.P.) but not from the action of laked blood corpuscles. These experiments are summarized in Table III. 15 units of insulin were incubated with 1 c.c. of 1 per cent. pancreatin in a buffered solution (pH 7·6) for two hours at 37° C., and 10 c.c. aliquot portions from the total volume of 50 c.c. were injected intravenously into 4 rabbits; the same animals were, at another time, treated with a similar mixture to which 10 units of antitrypsin had been added before incubation. Another group of 7 animals received similar amounts of insulin which had been incubated under similar conditions with 5 c.c. of laked diabetic red blood-cells, both with and without antitrypsin; control experiments with insulin plus saline were carried out on this group.

TABLE III.—*Average Effect of the Intravenous Injection of 3 Units Insulin Incubated with Pancreatin or with Laked Cells, with and without Antitrypsin.*

Injected mixture.	Blood-sugar (mgm. per 100 c.c.).				Convulsions.
	Initial.	60 mins.	120 mins.	180 mins.	
Insulin, pancreatin	120	122	124	122	None.
Ditto, plus antitrypsin	121	57	52	56	2 out of 4.
Insulin, laked cells	116	112	121	124	None.
Ditto, plus antitrypsin	121	111	119	121	,,
Insulin, saline	116	54	67	83	3 out of 7.

Some workers have considered that the leucocytes may be responsible for destruction of insulin within the circulation (Rosenthal and Behrendt, 1926; Karelitz, Cohen and Leader, 1930; Zeckwer, 1932). It was possible to obtain a sufficient quantity of leucocytes for investigation from a diabetic patient with severe leucocytosis. It was found that unlysed leucocytes suspended in a buffered solution containing insulin caused no detectable inactivation (of 3 units in 2 hours at 37° C., pH 7·6) as tested by intravenous injection into rabbits. After cytolysis, however, inactivation of insulin similar to that obtained with laked red corpuscles may be demonstrated. No experiments were made with leucocytes from non-diabetic subjects; but it appears to be certain that the inactivating substances within the cells are powerless to affect insulin present in the plasma.

INACTIVATION OF INSULIN BY WHOLE BLOOD.

The chief purpose of this investigation being to examine the alleged differences between diabetic and normal blood, it was felt desirable to carry out some experiments under conditions more physiological than those recounted above. Diabetic blood was obtained from a patient who was considered insulin-resistant in that, in spite of his receiving 100 units daily, his fasting blood-sugar was high (323 mgm. per 100 c.c.), and he was excreting 50 gm. of glucose daily. The blood (approximately 60 c.c.) was collected in a flask containing 15 mgm. heparin; the flask was immediately carried to the laboratory in a vessel of water warmed to 38–40° C., and 49 c.c. of the heparinized blood was transferred to a flask which was kept agitated in a water-bath at 37° C., to which 1 c.c. of saline containing 15 units of insulin was added. The mixture was incubated for 2 hours, and 10 c.c. aliquots were injected intravenously into fasting rabbits. Normal blood was treated and examined in

TABLE IV.—*Average Effect of the Intravenous Injection of 3 Units Insulin Incubated with Heparinized Whole Blood.*

Blood.	Blood-sugar (mgm. per 100 c.c.).			
	Initial.	60 mins.	120 mins.	180 mins.
Normal	127	47	70	92
Diabetic	124	47	54	83
Control	117	49	76	103

the same way, and control experiments with saline in place of blood were also carried out; a group of 5 rabbits was used for these experiments. It appears from the table that neither the normal nor the diabetic blood had any appreciable destructive effect upon the insulin under these conditions.

It may be concluded from these and the preceding experiments that while red blood-corpuscles and leucocytes undoubtedly contain substances, probably enzymes, capable of inactivating insulin, these substances do not escape from the cells to affect insulin in the plasma with any facility. The insulin-inactivating power of serum has not been investigated by the intravenous injection method; the experiments with whole blood indicate that it would probably be found to be trivial. Above all, the experiments as a whole lend no support to the theory that diabetic blood is more capable of destroying insulin than is normal blood.

SUMMARY.

1. Insulin incubated with serum or with laked cells or with cytolysed leucocytes loses some part of its power to lower the blood-sugar and produce convulsions in fasting rabbits when the mixture is injected subcutaneously.

2. Insulin incubated with laked cells loses some part of its power to lower the blood-sugar when injected intravenously into rabbits. This is probably due to destruction of the insulin by an enzyme other than trypsin. The inactivating substance does not readily pass from the cells to the plasma.

3. Insulin incubated with heparinized whole blood retains its power to lower the blood-sugar when injected intravenously into rabbits. This is also true of insulin incubated with intact red cells or leucocytes.

4. Blood from diabetic patients, even from those displaying resistance to insulin, had no more than normal power to destroy insulin in any of these experiments. This investigation therefore does not support the view that the symptoms of diabetes are due to destruction of insulin in the circulation.

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THE TITRATION OF ANTI-PNEUMOCOCCUS SERUM (TYPE I) IN MICE.

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THE following account of experiments on the titration of anti-pneumococcus serum (Type I) in mice represents an extension of the work we have already published on a quantitative analysis of the lethal power of a strain of Type I pneumococcus and on the curative action of anti-pneumococcus serum (Type I) in mice (Petrie and Morgan, 1931, 1932). The present communication contains: (1) further observations on the lethal character of the strain of pneumococcus that we have used as a test-culture from the commencement of our work; (2) an account of experiments designed to ascertain the degree of accuracy with which it is possible to estimate the protective antibody in anti-pneumococcus serum (Type I) by the particular method of titration we employ; and (3) observations that demonstrate the superiority in routine and experimental work of a stable standard—the recently introduced British standard serum—as compared with a liquid serum, even when this is maintained under the best available conditions of storage.

FURTHER OBSERVATIONS ON THE LETHAL ACTION OF A STRAIN OF TYPE I PNEUMOCOCCUS IN MICE.

The Technique of the Experiments.

The account of the technical details that has been already published absolves us from the necessity of giving a description of the methods used in the tests. Mice from several stocks were mixed at random for each test, and the observations on them were made every 4 hours for a period of 100 hours, so that the mean death-time of a group of mice may be assumed to be correct to $\pm$ 2 hours; groups of from 10 to 50 mice were used for each dose of culture.

The virulent strain that is used as a test-culture is passaged weekly through mice. It is convenient to choose one of the mice that dies early in the control group which receive $1/(1000 \times 10^6)$ c.c. of the test-culture. The thorax of the mouse is well seared after reflection of the skin, and then punctured with a capillary pipette over the position of the heart, so as to obtain blood

or, it may be, pleural effusion. The material is transferred to a tube of the "stock-culture" broth that is used for storing the culture. This broth consists of a medium, of which a description follows, that is similar to the one used for the test-culture, except that the horse-serum is omitted, and that 10 per cent. of the red cell deposit from fresh oxalated horse-blood is added to it. The heart-blood culture from the mouse is incubated overnight and then stored at room temperature in the dark. When an 8-hour culture is required for an experiment, a culture is first made into the special broth medium and grown overnight, and a second subculture is then made from this into the same medium.

Factors that Influence the Lethal Power of the Test-culture.

These have been discussed elsewhere at some length (Petrie and Morgan, 1931), and it was noted that the lethal power of a virulent pneumococcus culture is compounded of two factors: (1) the density of the culture, that is, the number of virulent diplococci contained in 1 c.c., and (2) the virulence of the strain; this may be regarded as constant if frequent passages through mice are made. The density of the test-culture is largely determined by the composition of the broth medium in which it is grown. An approximate estimate of the density counts of the cultures examined in our early experiments is 500,000,000 diplococci per c.c. During the period covered by the present series of tests the mean density is three or four times as high; the data on which this statement is based were obtained from the examination of cultures that were grown in two batches of broth, namely, Nos. 14 and 19. The routine practice was adopted of estimating the density of the test-culture by plate counts immediately before the mice were inoculated with the doses of culture. Each of 5 agar plates was sown with 2 c.c. of an equal mixture of normal horse-serum and a $1/(5 \times 10^6)$ dilution of the test-culture in broth. The total count represented the number of viable diplococci in $1/(1 \times 10^6)$ c.c. of the culture; hence the number per c.c. was readily calculated.

The mean of the density figures in 33 experiments that were carried out weekly on the titration of sera, as described in this paper, is 1,722,000,000 diplococci per c.c. when the culture was grown for 18 hours at 37° C. in medium No. 14. The mean of the counts in 15 tests, when the culture was grown in medium No. 19, is 2,239,000,000 diplococci per c.c. The stock tubes of both media were kept at room temperature, and it seemed that medium No. 14 improved during the whole period of storage, that is, 9 months, because the average count was higher in the last 17 than in the first 16 tests.

The batches of culture medium Nos. 14 and 19 were prepared as follows:

The broth medium No. 14 was made on June 7th, 1932, and was regularly used until March 18th, 1933. Two pounds of minced horse-meat, 2000 c.c. water, and 0·5 per cent. acetic acid were brought to 95° C., and filtered through muslin after neutralizing with soda. Two per cent. Difco proteose peptone was added, the pH was adjusted to 7·6 and the insoluble phosphates were allowed to settle. Five per cent. normal horse-serum was added to the supernatant fluid, and the mixture was filtered through a Pasteur-Chamberland F filter, tubed, and heated for one hour on each of two days at 60° C.

The broth medium No. 19 was prepared on November 11th, 1932, according to the same procedure as that which was followed in making medium No. 14, except that the meat infusion was filtered through chain-cloth before neutralizing the acid, and that a Berkefeld filter was used instead of the Pasteur-Chamberland filter. This broth was brought into use when the stock of No. 14 was exhausted, and served as the medium for the test-culture from April 20th to June 26th, 1933.

We believe that it is important to choose a medium which provides suitable conditions for the growth of the test-culture that is used for titrating the serum, and yet it is not easy to define the requisite factors. Attempts to reproduce batches of medium similar to broths Nos. 14 and 19 that will give consistent and satisfactory results are not always successful. A simple criterion of suitability is afforded by the routine microscopical examination of the culture by Gram's stain; this should show diplococci, with only a minor degree of chain formation.

The consistently high density of the later cultures is confirmed by the results of giving graduated doses intraperitoneally to groups of mice (Table I).

TABLE I.—*Showing the Relation, as Expressed in Terms of the Mortality-rate and the Death-time, Between Dosage of the Test-culture of Type I Pneumococcus Used in the Experiments and the Resulting Infection.*

Volume of culture injected (c.c.).	Number of experiments in group.	Average number of diplococci per c.c.	Calculated number of diplococci in dose.	Mice.			
				Number inoculated.	Number of deaths.	Percentage mortality.	Mean death-time (hours).
Batch No. of culture medium—14.							
1/(1000)	2	2390×10^6	**2,390,000**	99	99	**100**	35
$1/(10 \times 10^6)$	8	1712×10^6	**17**	190	184	**97**	44
$1/(200 \times 10^6)$	11	1679×10^6	**8·4**	110	104	**95**	48
$1/(400 \times 10^6)$	6	1489×10^6	**3·7**	60	49	**82**	50
$1/(500 \times 10^6)$	8	1688×10^6	**3·4**	110	84	**76**	53
$1/(800 \times 10^6)$	2	2200×10^6	**2·8**	100	83	**83**	55
$1/(1000 \times 10^6)$	15	1795×10^6	**1·8**	190	137	**72**	53
$1/(3200 \times 10^6)$	2	2200×10^6	**0·7**	100	38	**38**	54
Batch No. of culture medium—19.							
$1/(800 \times 10^6)$	1	2207×10^6	**2·8**	50	48	**96**	64
$1/(1000 \times 10^6)$ (8 hours' growth)	4	1896×10^6	**1·9**	60	48	**80**	52
$1/1000 \times 10^6)$ (16 hours' growth)	10	2322×10^6	**2·3**	100	65	**65**	61
$1/(3200 \times 10^6)$	1	2207×10^6	**0·7**	50	20	**40**	66

A comparison between this table and a similar table (XI) in the previous account (Petrie and Morgan, 1931) shows that the mortality-rates are uniformly higher in the observations that are recorded in Table I. One method of estimating the density of different cultures is to sow 1 c.c. of high dilutions of the cultures into serum-broth tubes, and note the proportion of those in which growth takes place. Table II affords a comparison between the results obtained by this method when the strain was inoculated into different batches

of broth, in some of which the culture grew with a low density and in others with a high density. The table shows further that, in agreement with the early experiments, mice, when they are inoculated with precisely the same amount of the same culture-dilution as was implanted in a series of serum-broth tubes, show a smaller percentage of positive results than those given by the culture-tube method ; the reason for this is not at present clear.

TABLE II.—*A Comparison between:* (1) *the Results of Injecting Mice with the Proportion of Positive Growths in a Series of Culture-tubes*

Batch number of culture medium.	Volume of culture inoculated (c.c.).	Number of experiments in group.	Average number of diplococci per c.c.	Calculated number of diplococci in dose.
6, 7, 8, 9	$1/(800 \times 10^6)$	8	500×10^6	0·6
14	$1/(800 \times 10^6)$	2	2225×10^6	2·8
19	$1/(800 \times 10^6)$	1	2207×10^6	2·8
6, 7, 8, 9	$1/(3200 \times 10^6)$	6	500×10^6	0·2
14	$1/(3200 \times 10^6)$	2	2225×10^6	0·7
19	$1/(3200 \times 10^6)$	1	2207×10^6	0·7

THE TITRATION OF ANTI-PNEUMOCOCCUS SERUM (TYPE I) IN MICE.

The Technique Employed in the Tests.

The intravenous technique, of which a description follows, was devised by us independently of other workers in February, 1930, and has been adhered to up to the present time. Two methods may be used : in the one the serum serves as a prophylactic, and in the other as a curative agent.

The Prophylactic Method.

We regularly employ this method for both routine and experimental work. It consists in preparing suitable dilutions of the serum under test so that varying doses are contained in a constant volume of 0·5 c.c. ; these are injected intravenously into groups of ten, or multiples of ten, mice. One hour later, a dose of 1/1000–1/3000 c.c. of an 8–18-hour horse-serum-broth culture of the virulent strain, contained in a volume of 0·5 c.c., is injected intraperitoneally into the test animals. The mice that become infected are observed every 4 hours from the beginning of their illness until death, and the period of the observations is 100 hours from the time of injecting the test-dose of the culture. The term " survival-time " is used in this paper to denote the time that elapses between the injection of the culture-dose and the hour of death in serum-treated mice ; and it corresponds to the term " death-time " which we have applied to mice that are inoculated with the culture alone. In routine titrations of sera obtained from horses during the course of immunization, a dose of the British standard serum which contains one unit is given to each animal in a control group of at least 20 mice ; reference to this standard unit will be found below. Equivalence of the mortality-rate of the mice in this group and any other group in the test indicates that the corresponding dose of the serum under test contains one unit. At least two doses

of the serum that is being tested are administered to groups of mice, and the final judgment of its protective value is influenced by the consistency of the results. The dose-intervals of the serum are arranged with reference to the degree of closeness of testing that is required and to the number of mice in each group. The choice of the dose of the test-culture depends upon the particular batch of broth medium in which it is grown and the time of growth, and an endeavour

Minimal Doses of the Virulent Culture when Grown in Different Media ; and (2) *Sown with the Same Amount of the Same Dilution of the Culture.*

Mice.				Serum-broth tubes.		
Number inoculated.	Number of deaths.	Percentage mortality.	Mean death-time (hours).	Number inoculated.	Number yielding growth.	Percentage positive.
99	48	**48**	58·0	90	51	**57**
100	83	**83**	55·4	100	98	**98**
50	48	**96**	64·2	50	50	**100**
220	43	**19·5**	56·0	200	35	**17·5**
100	38	**38**	54·4	100	54	**54**
50	20	**40**	66·4	50	33	**66**

is made so to adjust it that one unit of the standard serum will protect about 50 per cent. of the control mice ; if this should be the result the test-dose contains, according to our data, approximately 600,000–800,000 living diplococci. In tests carried out in this way the serum functions as a prophylactic agent.

The Curative Method.

Experimental work on the curative action of anti-pneumococcus serum (Type I) has indicated that a method of titration of the antibody may be used by arranging for the intravenous administration of graduated doses of the serum to groups of mice at a suitable interval after a highly lethal dose of the culture has been given to them intraperitoneally (Petrie and Morgan, 1932). In these conditions the serum exerts a curative action and may bring about complete recovery, even when a bacteriæmia is present. A comparison of the results obtained by the prophylactic and by the curative method shows that they are equally capable of measuring the protective antibody in the serum. Nevertheless, the convenience of the prophylactic method has proved to be definitely advantageous in practice, for the injection of the doses of culture and serum in the curative tests as we arrange them cannot be disposed of within the ordinary working day. The proof that the prophylactic method furnishes a means of measuring the curative antibody confirms the belief that it is a suitable one for the titration of anti-pneumococcus sera.

The suggestion has been made that the difficulty of the intravenous technique in mice should be taken into account in judging the suitability of methods of titration. Our experience has been that, with practice, the intravenous technique is easy ; 120 mice can be inoculated intravenously in 60 minutes with the aid of an assistant who prepares the tails for injection. Failure to make a satisfactory injection is rare, even in mice with brown tails ; these, indeed, give uniformly successful results.

The Influence of the Sex and the Weight of the Mice upon the Results of Serum Titration Tests.

In our first paper (1931) the conclusion was drawn that the mean time of survival of male and female mice, when they were given equal doses of the virulent culture, is almost identical, and that the mortality-rate is almost equal in the weight-groups 15–19 gm. and 20–24 gm. inclusive. An examination has been made of data abstracted from tests in which the mice received a prophylactic dose of one unit intravenously and the usual intraperitoneal test-dose of culture one hour later. The results show that the survival-time of 146 male mice was 37·5 hours; of 101 female mice it was 38·8 hours. In the same set of experiments, 87 mice in the weight-group 15–19 gm. inclusive survived 39·2 hours; and 140 mice in the group 20–24 gm. inclusive survived 37·6 hours. It seems, then, that weight within the limits mentioned and sex are not correlated with a factor of susceptibility or resistance when the antibody fails to avert death from an infecting dose of culture of the order used in this investigation.

The Serum Reagents Used in the Tests.

Four serum reagents have been tested: (1) The reconstructed British standard serum; (2) the five-fold dilution of this standard in 66 per cent. glycerol-saline, which is distributed from the National Institute for Medical Research, London, as a convenient and stable form of the standard; (3) the serum F. 146, obtained through the courtesy of Dr. Lloyd D. Felton, Harvard; and (4) a glycerinated concentrated preparation which we employ as a laboratory standard. A full account of the two British standard preparations and of estimates of their potency in terms of serum F. 146 will be found in a recent paper by Hartley, Parish, Petrie and Wilson Smith (1933). It will suffice to state here that: (1) the British provisional unit is contained in 0·0886 mgm. of the dry stable standard preparation; (2) 1/1000 c.c. of the reconstructed dry standard serum contains one provisional unit; and (3) 1/200 c.c. of the glycerol solution contains one provisional unit. This provisional unit has been based on the unit that was originally proposed by Dr. Felton.

The Limits of Accuracy of the Intravenous Method of Serum Titration in Mice.

The prophylactic method was used throughout the tests.

Table III summarizes the results of six tests, which show that differences in the amount of antibody injected into the mice of + 25 per cent. and − 29 per cent. from an intermediate ratio can be detected when each dose is inoculated intravenously into groups of 50 mice. The protective value of the British standard reagents was chosen for comparison, and the test-dose contained from one to two million living diplococci. Since the glycerinated standard is obtained by making a five-fold dilution in glycerol-saline of the reconstructed dry standard, we should expect that adjustment of the dose of each reagent in the ratio 1 : 5 will result in approximately equal mortality-rates, and it is

TABLE III.—*Showing the Results of Comparing the Two British Standard Reagents by the Prophylactic Method; the Ratio of the Doses varied from 1/5 to 1/4 and 1/7, that is, a Difference from the Intermediate Ratio of + 25 per cent. and — 29 per cent.*

No. of experiment.	Serum doses. Ratio of volumes of doses. R.B.D.S./B.G.S.*	Percentage difference from intermediate ratio.	Serum doses. Volume (c.c.). R.B.D.S.	Serum doses. Volume (c.c.). B.G.S.	Serum doses. Units in dose. R.B.D.S.	Serum doses. Units in dose. B.G.S.	Average number of diplococci in test-dose.	Results of tests. Group mortality. R.B.D.S.	Group mortality. B.G.S.	Percentage mortality. R.B.D.S.	Percentage mortality. B.G.S.	Mean survival-time (hours). R.B.D.S	Mean survival-time (hours). B.G.S.
141	1 : 4	+25	1/900	1/225	1·11	0·89	1,857,000	26/50	40/50	51	77	26	29
142			1/900	1/225	1·11	0·89		25/50	37/50			27	26
135	1 : 5	—	1/1500	1/300	0·67	0·67	1,450,000	42/50	43/50	71	74	25	25
137			1/1250	1/250	0·80	0·80		29/50	31/50			24	30
139	1 : 7	—29	1/1575	1/225	0·63	0·89	1,368,000	41/50	19/50	89	57	40	46
146			1/1400	1/200	0·71	1·00		48/50	38/50			34	41

* R.B.D.S. = Reconstructed British dry standard serum; B.G.S. = British glycerinated standard serum.

seen from the table that the deaths in each group—71 per cent. and 74 per cent.—confirm this belief (Experiments 135 and 137). The mortality-rates in the groups of mice that were given doses of the two reagents in the ratio 1 : 4 (Experiments 141, 142) and 1 : 7 (Experiments 139, 146), which represent differences of + 25 per cent. and — 29 per cent. from the ratio 1 : 5, not only deviate from the equal rates given by the 1 : 5 ratio, but show a complete reversal corresponding to the relative increase or decrease in the amount of antibody that was injected. It may be added that these results confirm the conclusion which is stated in the report on the British standards (Hartley *et al.*, 1933), that dilution of the reconstructed standard serum in 66 per cent. glycerol-saline has no deleterious action on the protective antibody that is titratable by the mouse test.

TABLE IV.—*Giving details of Experiments carried out by the Prophylactic Method with the Object of Determining the Degree of Accuracy of the Test. Each Dose-interval of Serum Represents a Difference of 25 per cent.*

Experiment No.	Dose of serum. c.c.	Dose of serum. Number of units in dose.	Average number of diplococci in test-dose.	Results of tests in mice. Number inoculated.	Number of deaths.	Percentage mortality.	Mean survival-time (hours).
			British Glycerinated Standard Serum (Natural Serum Preparation).				
150	1/160	1·25	654,000	40	9	22·5	44
	1/200	1·00	654,000	40	20	50·0	43
	1/267	0·75	654,000	40	32	80·0	45
151	1/160	1·25	515,000	40	13	32·5	48
	1/200	1·00	515,000	40	12	30·0	43
	1/267	0·75	515,000	40	22	55·0	55
152	1/160	1·25	773,000	40	19	47·5	40
	1/200	1·00	773,000	40	26	65·0	37
	1/267	0·75	773,000	40	35	87·5	36
156	1/178	1·125	940,000	40	24	60·0	48
	1/229	0·875	940,000	40	28	70·0	48
168*	1/888	1·125	853,000	40	30	75·0	28
	1/1143	0·875	853,000	40	37	92·5	31
169	1/178	1·125	646,000	40	15	37·5	47
	1/229	0·875	646,000	40	12	30·0	56
170	1/178	1·125	713,000	40	12	30·0	40
	1/229	0·875	713,000	40	22	55·0	45
			A Concentrated Glycerinated Serum.				
159	1/240	0·83	254,000	40	27	67·5	41
	1/300	0·67	254,000	40	29	72·5	36
	1/400	0·50	254,000	40	32	80·0	37
174	1/200	1·00	652,000	40	28	70·0	31
	1/250	0·80	652,000	40	30	75·0	26
	1/333	0·60	652,000	39	34	87·2	25

* The serum used in this experiment was the reconstructed British Dry Standard.

Table IV gives the details of seven tests that were carried out with the British standard serum and two tests with a glycerinated concentrated preparation ; the prophylactic method was employed. In some of the tests a central dose was selected, with the hope that it would result in a mortality-rate of approximately 50 per cent., and doses differing from the central dose by + 25 per cent. and — 25 per cent. were given to equal groups of mice. In the experiments as a whole, the dose of antibody that was injected

intravenously into the mice varied from 0·5 to 1·25 of the British provisional unit. When 3 doses were given, the central dose was invariably one unit; each dose was inoculated into a group of 40 mice. The table shows that out of 10 pairs of co-related doses of the British standard serum, the mortality-rates corresponding to 8 of the pairs are in the expected order, and that, similarly, the mortality-rates corresponding to each of the 4 pairs of doses of the gycerinated concentrated serum are in order.

Table V gives precisely similar data to those in Table IV, except that the dose-interval represents a difference of 12·5 per cent. from the central dose. The results show that out of 8 pairs of comparable mortality-rates, 6 are in agreement with the doses of antibody injected and 2 are discrepant.

Table VI sums the numerical data that are given in Tables IV and V, with the result that each group comprises 120 or 160 mice. When the 5 pairs

TABLE V.—*Giving Details of Experiments carried out by the Prophylactic Method with the Object of Determining the Degree of Accuracy of the Test. Each Dose-interval of Serum Represents a Difference of* 12·5 *per cent.*

Experiment No.	Dose of serum. c.c.	Dose of serum. Number of units in dose.	Average number of diplococci in test-dose.	Results of tests in mice. Number inoculated.	Number of deaths.	Percentage mortality.	Mean survival-time (hours).
British Glycerinated Standard Serum (Natural Serum Preparation).							
156	1/178	1·125	940,000	40	24	**60·0**	48
	1/200	1·000	940,000	40	23	**57·5**	50
	1/229	0·875	940,000	40	28	**70·0**	48
168*	1/888	1·125	853,000	40	30	**75·0**	28
	1/1000	1·000	853,000	40	36	**90·0**	29
	1/1143	0·875	853,000	40	37	**92·5**	31
169	1/178	1·125	646,000	40	15	**37·5**	47
	1/200	1·000	646,000	40	10	**25·0**	62
	1/229	0·875	646,000	40	12	**30·0**	56
170	1/178	1·125	712,000	40	12	**30·0**	40
	1/200	1·000	712,000	40	17	**42·5**	46
	1/229	0·875	712,000	40	22	**55·0**	45

* The serum used in this experiment was the reconstructed British Dry Standard.

TABLE VI.—*Summarizing the Results of the Experiments referred to in Tables IV and V.*

Experiment No.	Dose of serum. c.c.	Dose of serum. Number of units in dose.	Average number of diplococci in test-dose.	Results of tests in mice. Number inoculated.	Number of deaths.	Percentage mortality.	Mean survival-time (hours).
Tests in which each dose-interval of serum represents a difference of 25 *per cent.*							
150	1/160	1·25	647,000	120	41	**34·2**	44
151	1/200	1·00	647,000	120	58	**48·3**	40
152	1/267	0·75	647,000	120	89	**74·2**	44
156, 168, 169, 170	1/178	1·125	788,000	160	81	**50·6**	39
	1/229	0·875	788,000	160	99	**61·9**	42
Tests in which each dose-interval of serum represents a difference of 12·5 *per cent.*							
156, 168, 169, 170	1/178	1·125	788,000	160	81	**50·6**	39
	1/200	1·00	788,000	160	86	**53·8**	42
	1/229	0·875	788,000	160	99	**61·9**	42

of comparable doses are considered, it is found that the corresponding mortality-rates conform to expectation.

This section of the work may be summarized in the statement that the results of the tests show a satisfactory degree of correspondence with the dose of antibody administered, despite the variable factors that must influence the titration of a serum when the test-dose consists of a living culture of highly virulent bacteria of which one diplococcus—by its multiplication in the body—is capable of infecting and killing a mouse. The chief difficulty we have encountered is in correctly judging a central dose of antibody which, in conjunction with a particular test-dose of culture, will give a mortality-rate in a group of mice of approximately 50 per cent. In order to economize mice it is important, as we pointed out in our first communication, to gain as much knowledge as possible of the factors that determine the lethal power of the particular strain that is used for the test-culture. Control over these factors will doubtless simplify titration of the serum in mice.

THE QUESTION WHETHER REGULAR RESULTS ARE OBTAINABLE WHEN THE DOSE OF SERUM IS KEPT CONSTANT AND VARYING DOSES OF THE TEST-CULTURE ARE GIVEN.

It may be supposed that, if the intravenous method gives satisfactory results when a constant test-dose of culture is used, there is no reason why equally satisfactory results should not follow when the serum-dose is kept constant and the doses of the culture are varied. This type of experiment is the reverse of the one that has been described. Table VII and Fig. 1 present the summed results of three experiments of this kind. The limits of the test-doses of culture represent a tenfold range, and the mortality-rates, when plotted, fall on a smooth curve; these vary from 13·3 to 81·6 per cent. It is of interest to compare these results with those of Wilson Smith (1932); in his experiments, however, the serum-dose and the test-dose of the culture were injected intraperitoneally after remaining in contact for 5 minutes. Smith concludes that an increase in the dose of organisms of more than one hundredfold fails to produce any significant increase in the mortality-rate when this method of titration is employed.

Confirmation of the results shown in Table VII will be found in Table VIII, which groups a number of scattered observations on the effect of administering one unit intravenously, and one hour later the dose of the test-culture intraperitoneally. The mean figures representing the number of cocci in the test-dose were obtained by averaging doses of cocci that were of the same order of magnitude. The results again show excellent correspondence between the number of pneumococci inoculated and the resulting mortality-rate.

The results obtained in the two types of experiment, in which either the serum-dose or the culture-dose is kept constant, support the belief that the method of titration as carried out by us, namely, the intravenous injection of the dose of serum one hour before the intraperitoneal dose of culture, permits a satisfactory degree of accuracy in testing the antibody content of natural and concentrated anti-pneumococcus serum (Type I).

TABLE VII.—*Summarizing the Results of Three Experiments in which the Dose of Serum—One Unit of the British Standard Serum—was Kept Constant, and Varying Doses of the Test-culture were Given. The Dose of Serum was Given Intravenously One Hour before the Intraperitoneal Dose of the Test-culture.*

Test-dose (c.c.).	Number of diplococci in test-dose.	Results of tests in mice.			
		Number inoculated.	Number of deaths.	Percentage mortality.	Mean survival-time (hours).
1/1000	**2,051,000**	60	49	**81·6**	41
1/2000	**1,025,500**	60	42	**70·0**	42
1/3000	**683,700**	80	32	**40·0**	40
1/5000	**410,200**	60	12	**20·0**	54
1/10,000	**205,100**	60	8	**13·3**	41

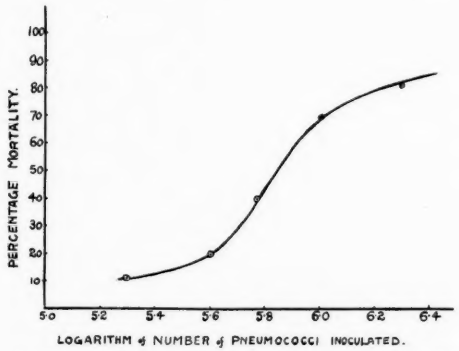

FIG. 1.—The curve shows the relation between the mortality-rates and the logarithms of the number of pneumococci in varying doses of the test-culture when given to groups of mice which received an intravenous dose of serum containing one British unit.

TABLE VIII.—*A Summary of the Results of Experiments in which One Unit of the British Standard Serum was Given Intravenously to Mice, and One Hour Later Varying Doses of the Test-culture were Injected Intraperitoneally.*

Batch number of culture medium.	Number of experiments in group.	Average number of diplococci in test dose.	Results of tests in mice.			
			Number inoculated.	Number of deaths.	Percentage mortality.	Mean survival-time (hours).
19	9	717,000	240	118	49·2	41
14	4	746,000	160	81	50·6	43
14	5	1,467,000	230	162	70·4	38
14	1	2,516,000	50	49	98·0	37

THE RELATION BETWEEN THE MORTALITY-RATES AND THE MEAN SURVIVAL-TIMES OF SERUM-TREATED MICE WHICH DIED.

When varying doses of the test-culture alone are given to groups of mice, the interval which elapses between the time of injecting the dose and the time

of death decreases as the number of lethal doses increases above the $1/(10 \times 10^6)$ c.c. dose (Table XI, Petrie and Morgan, 1931, and Table I, *supra*). As we have already pointed out, the close correspondence between the data which define the course of the infection in mice that receive a dose within the range of $1/(3200 \times 10^6)$ c.c. and $1/(16 \times 10^6)$ c.c. of a culture with a mean density of 500,000,000 diplococci per c.c., suggests that the numbers (1–50) of diplococci in doses within this range possess the same lethal action; the mean death-time varies from 48 to 58 hours.

The mean death-time of groups of mice which were inoculated with the test-dose of the culture (1/1000 c.c.) was found to vary from 32 to 35 hours, and most of the mice died within the 8-hour period corresponding to 21–28 hours from the time of injecting the dose of culture; a total of 169 mice received this dose.

A reduction in the mortality-rate of a group of serum-treated mice, as compared with a control group which has received the test-dose of culture only, is evidence of its beneficial action, but the question may be asked whether the serum prolongs life in the mice that ultimately succumb to the infection. Table XI contains an analysis of 1780 serum-treated mice

TABLE XI.—*Showing the Relation between the Mortality-rates and the Mean Survival-times of Serum-treated Mice which died.*

	Mortality-rate (per cent.).								
	20–29.	30–39.	40–49.	50–59.	60–69.	70–79.	80–89.	90–99.	100.
Mean survival-time	53·3	46·5	43·5	38·2	39·3	33·7	34·1	34·0	31·1
Number of mice	24	109	56	212	266	360	322	361	70

which died. The data are grouped according to the mortality-rates, and the figures show that the mean survival-time for each group of mice bears on the whole an inverse relation to the corresponding death-rate—a result which indicates that the serum, although it was powerless to avert death, had the effect of prolonging the struggle against the infection. Further, the survival-times, when expressed in hours, of 1758 serum-treated mice that died have been distributed in successive 4-hourly periods from the time of infection to death for each mortality-rate from 20 per cent. to 100 per cent. at intervals of 10 per cent. When this was done, it was found convenient to sum the data corresponding to mortality-rates from 20 per cent. to 49 per cent. and to re-group the whole of the survival-times in 8-hourly periods. The results of this analysis are briefly as follows:

When the mortality-rate is 49 per cent. or less, the 8-hourly period within which the greatest number of mice died is 37–44 hours, whereas when the mortality-rate is 50 per cent. or more, the 8-hourly period within which the greatest number of mice died is the next shorter period, namely, 29–36 hours; this relation holds for the data corresponding to each increment of 10 per cent. above the 50 per cent. mortality-rate. Now, as already stated, most of the mice which received the test-dose of culture alone died within the period

21–28 hours, which again is the next shorter 8-hour period. These results confirm the conclusion mentioned above that the serum prolongs life, even in the mice which eventually died. Curves, of which there are eight in all, have been constructed for each set of distributions. They present a striking regularity, and possess two features that are apparent on inspection : First, there is the regular succession of the curves, depending upon whether the culture was given alone, and upon the degree of protection conferred by the serum, as measured by the mortality-rates ; and secondly, the curves show the proportionately increasing number of mice whose survival-times fall within the 29–36-hour limits, as the mortality-rate increases from 50 per cent. to 100 per cent. The analysis as a whole makes it clear that, although death may ensue, the length of the illness of any mouse is determined, on the average, by the degree of protection conferred by the serum. The tables that accompany this paper furnish instances which appear to be contradictory to this conclusion, because the survival-times are sometimes alike in groups of mice with mortality-rates that differ considerably. These results are almost certainly due to sampling errors from the relative smallness of the groups, and are not, therefore, opposed to the conclusion we have reached. A final comment may be made. The distribution curves show unmistakably that the limit of observation—namely, 100 hours—chosen for the particular method of titration employed by us, corresponds to a time when the curves are merging into the base-line, that is, when the mortality-rate is insignificant.

OBSERVATIONS THAT ILLUSTRATE THE IMPORTANCE OF EMPLOYING IN ROUTINE AND EXPERIMENTAL WORK EITHER THE DRY STABLE STANDARD PREPARATION OF ANTI-PNEUMOCOCCUS SERUM (TYPE I) OR THE GLYCEROL-SALINE SOLUTION DERIVED FROM IT.

In the early months of 1932 we took part in a joint inquiry to determine the potency of the British standard serum in terms of the liquid standard serum F. 146. An account of this inquiry, which gives the experimental results in a condensed form, will be found in the article by Hartley *et al.* (1933).

From March to November, 1932, we carried out comparative tests on these reagents by both the prophylactic and the curative method. The tests that were performed during the first five months of the period gave consistent results, which pointed to a potency-ratio of 1 : 5, the reconstructed standard being five times more potent per unit volume than serum F. 146. Table IX summarizes the results of these tests. The difference in the mortality-rates in the curative experiments of May and June, when the ratio of the doses was 1 : 5, is not statistically significant. The later results were, however, not consistent with the 1 : 5 potency-ratio (Table X). Thus curative experiments that were carried out in August, 1932, with doses adjusted to give a ratio of 1 : 7, yielded results that indicated equivalent antibody content in the two preparations. Further, when the prophylactic type of experiment was repeated in September and October, 1932, with a dose-ratio of 1 : 5, the results did not suggest equivalence of antibody, because the mortality-rate was considerably greater in the group of mice that received serum F. 146

TABLE IX.—*Summarizing the Early Prophylactic and Curative Tests that Standard Serum*

Type and date of experiment.	Number of experiments in group.	Ratio of volumes of doses.	Serum doses. R.B.D.S. c.c.	R.B.D.S. Units.	Serum F. 146. c.c.	Serum F. 146. Units.
Prophylactic (March and April, 1932)	3	1:5	1/2000	0·50	1/400	0·50
			1/1000	1·00	1/200	1·00
Curative (May and June, 1932)	5	1:5	1/200 to 1/800	5·00 to 0·80	1/40 to 1/160	5·00 to 0·80
Curative (July, 1932)	2	1:4	1/240	4·17	1/60	3·33

TABLE X.—*Summarizing the Later Prophylactic and Curative Tests that were Serum and Serum F. 146. The Results indicate that the*

Type and date of experiment.	Number of experiments in group.	Ratio of volumes of doses.	Serum doses. R.B.D.S. c.c.	R.B.D.S. Units.	Serum F. 146. c.c.	Serum F. 146. Units.*
Curative (August and Septemper, 1932)	2	1:3	1/180	5·55	1/60	3·33
			1/180	5·55	1/60	3·33
Prophylactic (September and October, 1932)	4	1:5	1/2000	0·50	1/400	0·50
			1/1000	1·00	1/200	1·00
			1/1000	1·00	1/200	1·00
			1/750	1·33	1/150	1·33
Curative (August, 1932)	2	1:7	1/420	2·38	1/60	3·33
			1/420	2·38	1/60	3·33
Prophylactic (November, 1932)	2	1:7	1/1225	0·82	1/175	1·14
			1/1225	0·82	1/175	1·14

* Calculated on the assumption that serum F.146 had not diminished in potency.

than in those which received the British standard. Lastly, a prophylactic experiment which was carried out in November, 1932, with a dose-ratio of 1 : 7, gave virtually identical mortality-rates. The later results as a whole confirm each other, and show that towards the end of the year the sample of the liquid standard serum had lost about 30 per cent. of the units it contained when the tests were begun.

This experience has impressed us with the importance of using a suitable control serum in routine and experimental work on the titration of anti-pneumococcus sera. This serum must fulfil the requirements for a stable standard preparation—that is, maintenance in such a form and under such physical conditions as will ensure that its antibody content, when expressed in terms of a strictly defined unit, for example, a definite weight of the dry preparation,

were carried out to Determine the Potency-ratio of the Reconstructed British and Serum F. 146.

Test-dose or infecting-dose of culture.		Results of tests.					
		Group mortality.		Percentage mortality.		Mean survival-time (hours).	
c.c.	Average number of diplococci.	R.B.D.S.	Serum F. 146.	R.B.D.S.	Serum F. 146.	R.B.D.S.	Serum F. 146.
1/1000	700,000	15/30	15/30	**50·0**	**50·0**	39	39
1/1000	700,000	11/40	9/30	**27·5**	**30·0**	47	43
1/20,000,000	53	52/120	44/120	**43·3**	**36·7**	46	44
1/10,000,000	184	34/100	49/100	**34·0**	**49·0**	57	56

carried out to Determine the Potency-Ratio of the Reconstructed British Standard Unitage of the Liquid Serum F. 146 had decreased.

Test-dose or infecting-dose of culture.		Results of tests.					
		Group mortality.		Percentage mortality.		Mean survival time (hours).	
c.c.	Average number of diplococci.	R.B.D.S.	Serum F. 146.	R.B.D.S.	Serum F. 146.	R.B.D.S.	Serum F. 146.
1/(10 × 10⁶)	181	19/50	27/50	**39·0**	**53·0**	60	54
1/(10 × 10⁶)		20/50	26/50			50	50
1/1000	1,549,000	41/50	46/50	**57·5**	**69·0**	39	40
1/1000		25/50	33/50			40	45
1/1000		33/50	39/50			33	34
1/1000		16/50	20/50			48	45
1/(10 × 10⁶)	189	31/50	36/50	**57·0**	**62·0**	49	44
1/(10 × 10⁶)		26/50	26/50			49	45
1/1000	1,547,000	34/50	34/50	**71·0**	**70·0**	40	41
1/1000		37/50	36/50			32	30

shall remain absolutely unchanged. The British standard sera possess the attributes that are implied in this description, and we can testify to the undoubted advantage of working with these preparations.

SUMMARY AND CONCLUSIONS.

1. Further evidence has been obtained which emphasizes the importance of recognizing and endeavouring to control the factors that influence the lethal power of the test-culture used in the titration of anti-pneumococcus serum (Type I) in mice. The employment of a suitable medium for the test-culture and the ability to select an appropriate test-dose of the culture simplifies the work, and conduces to economy in the number of animals that are required.

2. The technique that we have employed in the titration tests recorded in this paper consists in giving to the mice graduated doses of the serum by the intravenous route, and one hour later a constant test-dose of the culture intraperitoneally. In this type of experiment the serum functions as a prophylactic agent. Either a prophylactic or a curative method may be used, because both appear to be equally capable of titrating the curative antibody in the serum, but the former method has the advantage of practical convenience.

3. The prophylactic method permits a satisfactory degree of accuracy in titrating the serum, since differences in the doses of the order of 25 per cent. or even 12·5 per cent. are frequently indicated by using groups of 40 mice for each dose of serum. When the method is reversed, that is, when the serum-dose is kept constant and the doses of culture are varied, regular results are also obtainable.

4. The titration of routine batches of serum should be effected by simultaneous comparison with a stable standard serum. The recently introduced dry British standard preparation (Hartley *et al.*, 1933) is maintained in physical conditions that ensure permanent constancy of its antibody content, as expressed in terms of a strictly defined unit. Most of the tests that are embodied in this paper were carried out with the British standard reagents.

5. The therapeutic action of the serum in the tests we have described is apparent, not only in a reduction in the mortality-rate of groups of serum-treated mice as compared with mice to which the test-dose of culture alone is given, but in the prolongation of the illness of the serum-treated mice that ultimately die. Thus an analysis of the survival-times of more than 1750 serum-treated mice which died shows that the serum, although it failed to avert death, had the effect of prolonging the struggle against the infection.

We have pleasure in expressing our best thanks to Dr. P. Hartley, Director of Biological Standards, National Institute for Medical Research, for his kindness in providing generous supplies of the standard reagents.

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THE ESTIMATION OF UREA BY UREASE METHODS IN FLUORIDE BLOOD.

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THE inhibitory effect of fluorine on urease activity is a subject which has forced itself on the attention of those interested in blood preservation for the purpose of chemical analysis, since the time when Sander (1923) suggested a mixture of sodium fluoride and thymol as a general anti-coagulant and preservative. The essential function of the fluoride was as anti-coagulant and preventative of glycolysis, while the blood was maintained sterile by the other component. More recently the collection of specimens in sterile vessels or venules has found much vogue in clinical work, and in such receptacles sodium fluoride alone is necessary to function in the same sense as did Sander's mixture. Either method maintains a blood sample for several days in an eminently suitable condition for blood-sugar estimation and many other types of analysis, but owing to a general inhibitory effect on enzyme action, such samples are useless where it is desired to employ urease methods for blood-urea determinations, or where enzyme activity residing in the blood itself has to be measured. For example Roe, Irish and Boyd (1927), and also Osterberg and Schmidt (1928), had shown that quantitative estimations of blood-urea cannot be made in the presence of fluoride, using the well-known methods of Cullen and Van Slyke, or Myers.

Many circumstances arise which make it desirable to find methods of rendering fluoride blood suitable for those analyses involving the enzymes, particularly in regard to the urease action in urea determinations. Occasionally, for example, it is requested that both urea and sugar be determined on the one blood specimen. Where conditions are such that there must be considerable delay before the analysis can be begun, fluoride, so essential to ensure the accuracy of the one determination, invalidates the other, unless alternative methods to those founded on urease activity can be substituted. There are times, too, when those who collect blood for laboratory examinations are forgetful of the appropriate use of anticoagulants, or at the moment of withdrawing a specimen disregard labelled instructions. One outcome of such errors is to cause to arrive at the laboratory a number of specimens requiring blood-urea determination in fluoride blood.

The simplest way of meeting this situation is, of course, to substitute alternative methods, either for blood-urea as already mentioned, or to perform the non-protein nitrogen estimation and make deductions based upon the figure for non-protein nitrogen thus ascertained; but it was hoped that a better

solution might be found by arriving at a means of treating fluoride blood in such a way as to permit enzyme activity within it. This accomplished, one might then proceed to ascertain how nearly fluoride could function as a universal anti-coagulant and preservative for sterile blood.

In attacking the problem the obvious line to take was to remove the fluoride ion by the formation of an insoluble fluoride. Indeed the suggestion had already been put forward by Roe, Irish and Boyd (1927) that magnesium might be used for this purpose, though it is not evident from their reference as to why magnesium should be superior to, say, barium or calcium. Initial experience showed, however, that it was inadvisable to use any of the common soluble salts of these metals, for the excess metallic ions that were necessary to ensure complete precipitation of the fluoride had themselves a deleterious effect upon the urease activity. Among less soluble compounds the peroxides suggested themselves. They show some tendency to decompose in aqueous solution to give off small quantities of hydrogen peroxide, which, so far from being harmful, had an accelerating effect upon the urease. As the metal was being used up to form insoluble fluoride, so would more pass into solution from the peroxide, and thus, it was possible that the peroxide would furnish adequate metallic ions without introducing the harmful effect of an excess.

Examination of the use of the peroxides of magnesium, barium and calcium showed that these could be used with partial success for the purpose in view, but the quantitative results were somewhat inconsistent, and it was impossible to guarantee 100 per cent. recovery of added urea. The evidence, taken as a whole, pointed to magnesium as being the most suitable element of those investigated, in that a slight excess appeared to be less toxic to the enzyme, so that it was deemed advisable to explore further among magnesium compounds for the most suitable form in which to use that element.

While engaged upon this stage of the investigation the writer's attention was drawn to the compound caffeine magnesium salicylate which a colleague, E. H. Watson,* had been preparing for quite another purpose. This compound, the preparation and properties of which are given below, seemed to fulfil all the requirements which experiment had shown to be necessary, and when fully investigated, was proved to be entirely satisfactory in all except one respect. It was impracticable to operate in an acid medium. Phosphate also inhibited the action of this compound, and so the method generally used in this laboratory for urea estimation, that of Patterson (1925), was slightly modified so as to take account of these findings. Practically any method could be used provided the incubation with urease is not carried out in the presence of acid or phosphate, but the technique actually used was as follows: 2 c.c. of distilled water were placed into a 6-in. by $\frac{5}{8}$-in. test-tube; 1 c.c. of blood was added, together with the requisite quantity of urease. The mixture was incubated at 45° C. for 20 minutes, after which 5 c.c. of a saturated solution of potassium carbonate, 3 gr. of solid anhydrous potassium carbonate, 5 c.c. of a saturated solution of potassium carbonate plus a few drops of caprylic alcohol were added in the order given, and the aëration continued in the usual way.

* Watson, E. H., Dispensary, St. George's Hospital, London, to whom the author is greatly indebted for information regarding this compound.

Work was begun using commercial urease preparations, but the presence of buffers and preservatives in these preparations completely obscured the effect of the caffeine compound, and so a urease preparation was made by the direct precipitation with acetone from an aqueous extract of soya beans, and for the purpose of estimating urea in fluoride blood, 50 mgm. of a mixture of equal parts by weight of dry urease powder thus obtained and caffeine magnesium salicylate were used during the incubation instead of the usual 25 mgm. of urease.

As Tables I and II show, this technique was eminently suitable for counteracting the effect of fluoride up to a concentration of 30 mgm. per c.c., or 3 per cent., and since the concentration of fluoride is never likely to exceed this amount, an accurate estimation of urea in fluoride blood can be obtained easily and readily, ensuring no more difficulty than in the ordinary estimation of blood-urea.

TABLE I.—*Estimation of Urea in an Aqueous Solution of Urea to which varying amounts of Sodium Fluoride have been added, using Commercial Urease Extract* (A), *and Urease prepared by the method given in the text* (B).

Amount in mgm. of sodium fluoride added per c.c.	Amount of urea found using 25 mgm. urease.		Amount of urea found using 50 mgm. of the mixture of urease B and caffeine magnesium salicylate
	A.	B.	
0	83	79	..
10	27	29	82
10	25	28	81
10	27	28	82
30	—	—	78
50	—	—	62
100	12	14	34
200	8	9	14

TABLE II.—*Estimation of Urea in Samples of Blood to which varying amounts of Sodium Fluoride have been added.*

Blood specimen No.	No anticoagulant period. Urea found mgm./100 c.c.	50 mgm. of urease mixture used in presence of fluoride.	
		Fluoride present mgm. per c.c.	Urea found.
I	32	10	32
I	32	20	33
I	32	30	30
I	32	50	27
I	32	100	18
II	20	12	19
III	56	20	57
IV	90	25	88
V	204	10	207
VI	324	20	317

The mechanism of the interaction of sodium fluoride with caffeine magnesium salicylate is not so simple as one would at first be led to assume, as the following considerations show, if the assumption be made that the organically combined magnesium merely replaces the sodium fluoride, thus :

$$(X)_2 \ Mg + 2 \ Na \ F \rightarrow Mg \ F_2 + 2 \ Na,$$

then 24·5 gm. of magnesium organically combined, should counteract not more than 84 gm. of sodium fluoride; or 1·84 mgm., the amount contained in 50 mgm. of caffeine magnesium salicylate, should counteract not more than 6·3 mgm. Reference to the tables will show that 25 mgm. of caffeine magnesium salicylate are capable of rendering non-effective between 20 and 30 mgm. of fluoride, that is to say, about six times as much as the maximum on this assumption. It is inferred, therefore, that the principal mechanism involved is one of adsorption.

ADDENDUM.

Caffeine Magnesium Salicylate.

Preparation.—Take 106 gm. of caffeine, 69 gm. of salicylic acid, and 15 gm. of magnesium hydroxide. Grind to a fine powder in a mortar. Add 300 c.c. of 45 per cent. alcohol and stir well. Stand for 10 minutes. Transfer to an evaporating dish, and dry in air oven at 105° C. Take the dried material, make into a thick paste with acetone, and allow to stand for 5 minutes. Squeeze between filter-paper to remove as much acetone as possible. Finally dry in the air oven at 110° C., and powder.

Properties.—The substance is pale pink soluble in water, giving a clear solution neutral to litmus, which yields a gelatinous precipitate with 5 per cent. potassium hydroxide, but no precipitate with dilute acids.

SUMMARY.

1. Means of treating fluoride blood to permit urease activity within it have been examined.

2. Magnesium is suggested as the most suitable metal for counteracting the inhibitory effect of fluoride, and in the form of caffeine magnesium salicylate is very suitable for this purpose.

3. The mechanism of the reaction involved in the use of this magnesium compound is briefly discussed.

My thanks are due to Dr. J. Patterson for his encouragement and advice during the course of this work.

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TRYPTOPHANE AND THE GROWTH OF BACTERIA.

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EVIDENCE is beginning to accumulate that tryptophane is as essential for bacterial growth as for animal growth. Burrows (1932) found that an acid hydrolysate of casein would not permit growth of *B. botulinus* until tryptophane was added, while Mueller, Klise, Porter and Graybiel (1933) found that a strain of *B. diphtheriæ* would only grow in an acid hydrolysate of casein and Liebig's extract after addition of tryptophane. Both findings followed from the knowledge that tryptophane is destroyed during acid hydrolysis. Fildes, Gladstone and Knight (1933), using a medium of known chemical constitution, showed that *B. typhosus* would not ordinarily grow in the absence of tryptophane, but that it could be trained to do so. Further Knight and Fildes (1933) noted that tryptophane must be added to a medium in order to allow growth of *B. sporogenes* in the presence of their sporogenes vitamin. The details of this latter observation are of technical interest in this type of work.

Attention was first directed to tryptophane by the discovery that one sample of glutathione had a remarkable effect upon the growth of *B. sporogenes*, while another had none at all. Bearing in mind the early methods for preparing glutathione, the active sample was tested for tryptophane and found positive. Tryptophane was then used instead of "glutathione", but the growth was poor. It is now understood that the activity of the "glutathione" was due to its content of tryptophane *and* of sporogenes vitamin (Knight and Fildes, 1933).

TRYPTOPHANE IN THE GROWTH OF *B. SPOROGENES*.

The effect of tryptophane upon the growth of *B. sporogenes* may be shown by using the acid hydrolysate of gelatine, vitamin and thioglycollic acid described by Knight and Fildes (1933):

Experiment 1: *Tryptophane in the Growth of* B. sporogenes.

Basal medium = Knight and Fildes (1933). Tryptophane 0·2 per cent.–0·1 c.c. Sporogenes vitamin SA_8F_{ii} diluted 1/10–0·5 c.c. Total volume 10·0 c.c.

Tube.	Medium.	Growth. Hours. 20.	Hours. 26.	Days. 2.	Days. 3.	Days. 4.
1	Basal medium	0	0	0	0	0
2	Ditto + vitamin	0	0	0	0	0
3	,, + tryptophane	0	tr.	tr.	tr.	tr.
4	,, + vitamin + tryptophane	+++	+++	+++	++	++

The trace of growth in Tube 3 is due to traces of sporogenes vitamin in the gelatine basal medium.

* Both working with grants from the Medical Research Council.

This experiment shows that a medium which is totally inadequate for growth may be made adequate merely by adding small quantities of highly purified tryptophane. In many other experiments, however, the result was not so sharply cut, traces of growth occurring in the presence of vitamin and absence of tryptophane, and in order to support the contention that tryptophane is essential, it must be concluded that undisclosed tryptophane was present in the medium in such cases.

Gelatine is, of course, reputed not to contain tryptophane, and even if traces are present they should be destroyed by acid hydrolysis (*cf.* Burrows and Mueller *et al.*), but in fact amounts of tryptophane recognizable chemically are present in gelatine hydrolysed with 10 per cent. acid (Experiment 2).

Experiment 2 : *Presence of Tryptophane in Gelatine.*

20 gm. Nelson's Photographic No. 1 Gelatine were hydrolysed with 10 per cent. H_2SO_4 for 3 hours at 130° C. The solution was then treated by the Hopkins-Cole method for the separation of tryptophane, and the precipitates collected, freed from mercury and concentrated to 20 c.c. This solution, equivalent to a 100 per cent. solution of gelatine, gave a slightly positive test with the glyoxylic reagent.

Experiment 3 : *Fractional Mercury Precipitation of Yeast Extract.*

An extract of yeast was precipitated fractionally by mercuric sulphate in sulphuric acid according to the Hopkins-Cole procedure for the preparation of tryptophane. Seven precipitates were obtained. After removal of the mercury the activity of the fractions was tested on *B. sporogenes* using as a basal medium an acid hydrolysate of gelatine after precipitation by the Hopkins-Cole method, reinforced with glucose and cysteine. The growths obtained were correlated with the glyoxylic test.

Tube.	Precipitate.	Tryptophane added.	Growth in 25 hrs.	Glyoxylic.
1	—	—	0	—
2	1	—	0	0
3	2	—	0	0
4	3	—	0	0
5	4	—	++	+ sl.
6	5	—	+++	+
7	6	—	+	+ sl.
8	7	—	0	0
9	1	+	+++	+
10	2	+	+++	+
11	3	+	+++	+
12	7	+	+++	+
13	—	+	±	+

Notes.—(*a*) The figure 0 in reference to growth is used as a convenience, but in all cases a trace of growth was observed.

(*b*) A trace of growth occurred in Tube 1 owing to traces of tryptophane surviving in the gelatine (to be referred to later), and traces of sporogenes vitamin therein. The heavy growths are due to tryptophane in the precipitates or added as such *plus* sporogenes vitamin adsorbed on the precipitates (*cf.* the feeble growth with tryptophane without a precipitate).

Evidence of the activity of tryptophane was further sought by fractional precipitation of yeast extracts, which, of course, contained tryptophane (Experiment 3).

Experiment 3 shows a close correlation between the activity of yeast preparations and their tryptophane content, but that tryptophane without the yeast is ineffective, as was the tryptophane without the gelatine We concluded that while tryptophane was necessary, so were other constituents of the yeast, of which one is now identified as the sporogenes vitamin.

TRYPTOPHANE IN THE GROWTH OF *B. BOTULINUS* AND *B. TETANI*.

The effect of tryptophane upon the growth of *B. botulinus* may be shown under exactly the same conditions as upon that of *B. sporogenes*, because the gelatine basal medium is adequate for this organism (National Collection of Type Cultures No. 750, heated for two hours at 82° C.; washed and heated spores from surface cultures). In the case of *B. tetani*, however, this is not the case. In addition to the basal medium described for *B. sporogenes*, a preparation of yeast must be added. This preparation is referred to later and is apparently free from tryptophane. At any rate the gelatine basal medium containing 0·5 c.c. per tube of this yeast preparation will not allow growth of *B. tetani* (strain TM 8, Fildes) until pure tryptophane has been added.

TRYPTOPHANE IN THE GROWTH OF *B. DIPHTHERIÆ* AND *B. ANTHRACIS*.

Neither of these organisms (laboratory strains) are capable of growing upon the gelatine basal medium. They require the further addition of yeast extract. Actually the basal medium used in this present case was that described by Fildes, Gladstone and Knight (1933) for *B. typhosus*. It contained salts and buffer, together with 13 amino acids, glucose and sporogenes vitamin. This medium does not, however, allow growth until yeast extract has been added. An ordinary autolysate or extract of yeast, of course, contains tryptophane, and therefore the extract was put through the Hopkins-Cole process with the idea of removing tryptophane. This "tryptophane-free" extract was then added to the basal medium and tested alone and together with tryptophane. In both cases the growth was found to be as good without added tryptophane as with, and it was concluded that either tryptophane was not necessary or that there was still tryptophane in the yeast. We recalled the fact that traces of growth could still be obtained in the absence of tryptophane from mercury precipitated gelatine (*vide* p. 244), and assumed that this process had left behind an amount of tryptophane sufficient to allow full growth. (It may be pointed out that these questions cannot be settled by dilution experiments, because there are other necessary factors in the yeast which are required in a higher concentration than tryptophane is.) An autolysate of yeast was then taken and precipitated with 50 per cent. alcohol. The filtrate concentrated and freed from alcohol was then saturated (about pH 6·5) with mercuric sulphate and heated for 2 minutes at 100° C. After overnight in the cold, the precipitate was removed and the filtrate freed from mercury and neutralized with soda. This yeast preparation is that referred to in connection with the growth of *B. botulinus* and *B. tetani*. The effect upon *B. anthracis* and *B. diphtheriæ* is shown in Experiment 4.

Experiment 4.—*Tryptophane in the Growth of* B. anthracis *and* B. diphtheriæ.

Both strains were laboratory strains. The inoculum for *B. diphtheriæ* was 1 drop of a suspension made by rubbing up in 10 c.c. saline a trace of an agar slope culture; for *B. anthracis* 1 drop of a high dilution of washed spores. Basal medium 8 c.c. = salts, amino acids, glucose, sporogenes vitamin and tryptophane-free yeast. Volume = 10 c.c. Tryptophane 0·2 per cent. Tubes sloped in the incubator.

Tube.	Inoculum.	Water (c.c.).	Tryptophane (c.c.).	Growth (days). 1.	2.	3.	6.
1	Anthrax	2	0	0	0	0	0
2	,,	1·9	0·1	0	tr.	+++	+++
3	Diphtheria	2	0	0	0	0	0
4	,,	1·9	0·1	0	++	+++	+++

This experiment shows that a medium which does not allow growth of these organisms can be rendered adequate by the addition of a small amount of tryptophane.

TRYPTOPHANE IN THE GROWTH OF *STAPHYLOCOCCUS AUREUS*.

This organism (a laboratory strain) also will not grow on gelatine, but requires the addition of yeast. The effect of tryptophane was shown in the manner indicated in Experiment 4.

In the presence of tryptophane growth occurred in 22 hours and was heavy in 43 hours. In the absence of tryptophane no growth was observed until 43 hours. The growth in tryptophane was easily continued in subculture in tryptophane, but the growth without tryptophane was only propagated successfully through two cultures without tryptophane. Continued growth of staphylococcus did not therefore take place in the absence of tryptophane, but nevertheless 2 serial liquid cultures grew to full opacity in the absence of tryptophane before subculture failed. If it is allowed that the whole of the material taken from the agar culture was tryptophane, it may be calculated that the amount which was carried over with the second positive culture was of the order of 10^{-12} gm. This abortive growth without tryptophane cannot therefore be ascribed to tryptophane carried over from the agar. An experiment

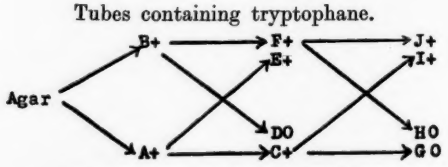

FIG. 1.

The letters indicate tubes; the arrows, subcultures; the *plus* signs, growth and "0", no growth. The growths in Tubes A and C were delayed 24 hours.

was then made in which subcultures were made according to the scheme in Fig. 1. Each inoculum from the liquid medium consisted of 1 drop of a 1 drop in 10 c.c. saline dilution. It will be seen that whereas the subculture from Tube A to Tube C grew, the subculture from Tube B to Tube D did not, although there was more tryptophane in B than in A. It seemed therefore obvious that the successful subculture from Tube A to C could not be due to the tryptophane content of the medium. It suggested an alteration in the quality of the bacteria in Tube A induced by a deficiency of tryptophane, similar to the alteration in *B. typhosus* described by Fildes, Gladstone and Knight (1933) whereby the bacteria acquired the power to grow in the absence of added tryptophane. In the example illustrated in Fig. 1 this new power was not sufficiently established to overcome the conditions of the experiment and permit of continued subculture, but in another instance when an inoculum from an old agar culture was used, the growth in the absence of tryptophane at first slow, soon acquired by serial subculture the power to grow as rapidly as with tryptophane. This culture has now been carried through 7 subcultures in the absence of added tryptophane. The sixth subculture was made in shallow flasks, and the mass of growth centrifuged and washed. A suspension of the growth was then tested for tryptophane according to the " aldehyde " method as described by Cole (1933), which in our hands is more sensitive than the " glyoxylic " method. As a control the bacterial suspension and sulphuric acid were used. A strong positive reaction was obtained, showing that the staphylococci contained tryptophane, which they must have synthesized from nitrogenous constituents of the medium. *Staphylococcus aureus* and *B. typhosus* are therefore similar in normally requiring tryptophane to be supplied in the medium, and in readily reacting to the absence of tryptophane by synthesizing it themselves.

TRYPTOPHANE IN THE GROWTH OF *B. TUBERCULOSIS* AND *B. AERTRYCKE*.

As is well known a number of " simple " bacteria can readily be grown in media of known composition in the absence of tryptophane, but that tryptophane has still to be considered in the metabolism of such organisms is shown by the fact that those which have been tested have been proved to synthesize tryptophane (Logie, 1919–20). We have found by Logie's method that *B. aertrycke*, which will grow readily on a medium containing ammonium chloride as the only source of nitrogen, synthesizes tryptophane.

In the case of *B. tuberculosis* we have examined a culture of a human type supplied to us by Capt. S. R. Douglas, which for years has grown in the absence of tryptophane and found tryptophane in the bacterial bodies. This organism therefore synthesizes tryptophane.

DISCUSSION.

The position of tryptophane in bacterial nutrition may be summarized as follows:

1. There is a group of bacteria which ordinarily cannot grow unless tryptophane is present in the nutrient medium : *B. sporogenes*, *B. botulinus*, *B. tetani*, *B. anthracis*, *B. diphtheriæ*, *B. typhosus*, *S. aureus*.

2. There is a second large group which does not require tryptophane to be present in the nutrient medium. This group includes all those organisms which will grow readily in simple media : Autotrophic bacteria, colon group, *B. pyocyaneus*, *B. aertrycke*, *B. tuberculosis* (?),* etc. Every member of this group which has so far been examined shows tryptophane in its protoplasm, namely various members of the colon group, *B. aertrycke*, *B. tuberculosis*. These organisms therefore are able to synthesize tryptophane.

3. The behaviour of a third group of organisms is of cardinal importance for the interpretation of the *rôle* of tryptophane. This group is intermediate between Group 1—the organisms which *ordinarily* do not grow in the absence of tryptophane, and Group 2, the organisms which grow *readily* in the absence of tryptophane. It consists of bacteria of Group 1, which are sometimes found spontaneously to behave as Group 2, or can be " trained " to do so ; *i. e. B. diphtheriæ* (Braun and Mündel, 1929), *B. tuberculosis* (?), *B. typhosus*, *S. aureus*. The last three organisms, when " trained ", have been shown to synthesize tryptophane.

It has thus been found that all bacteria examined either synthesize tryptophane or require it in their nutrient media, and it would appear probable that tryptophane is an essential chemical component of bacterial protoplasm.

Those bacteria which do not synthesize are dependent upon the preformed tryptophane present in animal or vegetable matter, whereas the others are able to synthesize tryptophane from inorganic sources. Thus there is a general tendency for the non-synthesizers to be found growing in living or dead animal tissues, while the synthesizers are more often found unassociated with animal tissues. It would appear that the parasitic or pathogenic habit is apt to be related to inability to synthesize tryptophane. It will, however, be observed that the synthesis of tryptophane is not an absolute property of a particular " species ". As are many other characters of bacteria, it is subject, at least in one direction, to controlled variation.

CONCLUSIONS.

1. Tryptophane is essential for the growth of certain bacteria, but some of these can be trained to grow without tryptophane.

2. All organisms which normally grow without added tryptophane, or can be trained to do so, synthesize tryptophane under these conditions.

3. Tryptophane is a constituent of all bacterial protoplasm.

4. There appears to be a relation between inability to synthesize tryptophane and pathogenicity.

The expenses of this work were defrayed by the London Hospital Research Fund and the Government Grant Committee of the Royal Society.

* Although *B. tuberculosis* was one of the first organisms to be grown upon a medium known to be free from tryptophane, there are no data to show whether this character is normal to the organism to the same extent as it is for instance to *B. pyocyaneus*. On general grounds, considering the difficulty of obtaining cultures as a routine direct from animal material, it may be doubted whether *B. tuberculosis* as a rule is capable of growing *readily* in the absence of tryptophane. We should prefer to look upon it as a member of the Group 3 following.

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THE INFLUENCE OF HISTAMINE AND PILOCARPINE ON THE HUMAN GASTRIC SECRETION.

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DURING recent years experimental work has been carried out by several workers on the hæmopoietic activity of gastric juice. In the majority of these experiments, employing human gastric secretion, histamine has been utilized to facilitate the collection of adequate amounts, on the assumption that it stimulates the secretion of both the acid and the enzyme fractions. Recent investigations, however (Babkin, 1930; Vineberg and Babkin, 1931), carried out on dogs have suggested that histamine only stimulates the production of hydrochloric acid, while pilocarpine influences the pepsin secretion. This indicated the possibility of serious fallacies if the histamine-stimulated gastric juice contained increased amounts of hydrochloric acid and only smaller residual quantities of pepsin and hæmopoietin (the gastric hæmopoietic principle), than would be anticipated if all the elements of the gastric juice were increased under its influence. A search through the literature revealed many discrepancies and omissions, and it appeared very desirable, therefore, to determine immediately the actual influence of histamine and pilocarpine on the secretion of gastric juice in man. The present communication is a short summary of part of our investigations with this object in view. The stimulating influence of histamine (subcutaneously administered) on the human gastric juice has been investigated by numerous workers using both the Ewald and fractional test-meals, and has led to its use as a clinical test for differentiating true and pseudo-achlorhydria. While there is general agreement that histamine provokes an excellent secretion of a gastric juice of increased acidity, opinion appears to be somewhat divided as to its influence on the enzyme elements of the gastric secretion. Polland and Bloomfield (1930) found that in normal patients the

total output of pepsin was increased following the injection of histamine, but that the concentration fell. Helmer, Fouts and Zerfas (1932) in 9 out of 10 healthy adults recorded an increase in the concentration of pepsin after histamine. On the other hand, Vineberg and Babkin (1931), using dogs with gastric pouches, concluded that histamine, while activating the secretion of water, hydrochloric acid and other inorganic constituents of the gastric juice, had no stimulating effect on the production of pepsin, but resulted in a marked decrease in the peptic power of the juice ; they believed that any pepsin present in the secretion following histamine stimulation had been merely washed out of the glands by the increased flow of fluid. They claimed, further, that pilocarpine injected subcutaneously specifically stimulated the production of the pepsin of the gastric juice, while the resulting secretion showed an increased volume, a slightly lowered acidity, and an enormous increase in the peptic power.

Gilman and Cowgill (1930, 1931) independently came to similar conclusions with regard to the influence of histamine. Polland (1932), however, using four human subjects, showed that successive doses of histamine always produced a definite rise in the total output of pepsin, amounting approximately to an increase of 100 per cent. The concentration was very variable, but always showed a fall during the height of secretion after the first stimulus. It is difficult to compare quantitatively the influence of histamine on the secretion of hæmopoietin owing to the enormous variation in the susceptibility of the individual cases of pernicious anæmia, these latter being necessary for the only test method (clinical) available for its detection. It can be definitely stated, however, from the experience of one of us (J. F. W.) that gastric juices obtained from normal individuals with and without the use of histamine appear to be sufficiently active, using equal quantities for suitable digestion experiments, to produce products that will give approximately equal responses. This point, however, is being determined more quantitatively, and will be reported later. Relatively little satisfactory information is available about the influence of pilocarpine on the gastric secretion, while considerable differences of opinion as to the type of juice evoked under its influence are revealed by an examination of the few papers dealing with it in the literature. That it effects a secretory response is clear, and, on the whole, the evidence is in favour of a juice of lowered acidity, but there is no consensus of opinion as regards its effect on the output of pepsin.

SUMMARY OF INVESTIGATIONS.

The subjects of this investigation comprised 88 individuals specially selected for their suitability for this work from the Infirmary in-patients. Of these, 32 complained of gastro-intestinal disturbances, although only 11 were shown to have had definite organic lesions (ulceration 10, appendicitis 1) ; 13 patients failed to secrete free acid under histamine stimulation. The remaining 43 did not complain of, nor did examination disclose, any such symptoms, and they apparently had normal gastro-intestinal tracts.

Each subject was prepared in the usual manner as for a fractional gastric analysis, and the entire contents of the stomach were withdrawn at the end of

each 10-minute interval. After the third sample had been aspirated histamine (0·25 mgm.), pilocarpine (6 mgm.), or both, were administered subcutaneously, and the aspirations continued at similar 10-minute intervals, each test lasting 2–3 hours. The usual precautions were taken to avoid contamination with saliva, while bile-stained samples were rejected and the test repeated. Each sample so obtained was measured, examined macroscopically, and the concentrations and total outputs determined for free hydrochloric acid, total acidities, total and mineral chlorides and pepsin.

The experiments were divided into the following groups according to the drugs used and the method of administration: (*a*) Normal controls without histamine or pilocarpine, (*b*) histamine alone, (*c*) pilocarpine alone, (*d*) histamine followed by a second dose of histamine 60 minutes later, (*e*) histamine followed by pilocarpine 60 minutes later, (*f*) histamine followed by histamine and pilocarpine simultaneously 60 minutes later, (*g*) pilocarpine followed by a second dose of pilocarpine 60 minutes later, and (*h*) pilocarpine followed by histamine 60 minutes later. Many of these cases were examined several times for comparative purposes, and accordingly fall into more than one of these groups.

GENERAL EFFECTS OF HISTAMINE AND PILOCARPINE.

In the present work histamine (0·25 mgm.) was conveniently used in the form of the acid phosphate, which was administered subcutaneously in doses of 0·75 mgm.

Particular observations were made on 50 cases as to the general effects following its administration.

In 2 patients the headaches present prior to the injection were increased in intensity. Five others complained of slight headache. In 12 patients no flushing was apparent, and in the remainder slight to marked flushing of the face and neck occurred.

Approximately half the cases showed an acceleration of the pulse-rate, varying from 6–12 beats per minute. In a few cases no alteration was noted, and in the remainder there was an increase of 18–30 beats per minute.

These effects commenced almost immediately following the injection of histamine, increased to a maximum in a few minutes and then passed off slowly.

Pilocarpine was used in the form of the nitrate, 6·0 mgm. being employed subcutaneously. The majority of the patients exhibited varying degrees of diaphoresis—in most of these it was of slight to moderate amount, but in several it was very profuse.

On four occasions there was an urgent desire to micturate, and one patient complained of abdominal pain. Usually there was no appreciable alteration in the pulse-rate, but a few cases exhibited slight acceleration, and in one case by as much as 30 beats per minute. The sialogogic action varied widely in different subjects from a slight to a profuse salivation. There did not appear to be any correlation between the influence of pilocarpine on the gastric and salivary secretions respectively.

The use of histamine and pilocarpine simultaneously in the same dosages gave systemic results additive in character, and there were a great number of

cases showing marked acceleration of the pulse-rate by more than 24 beats per minute. The greatest acceleration noted was 42 beats per minute in one case.

SUMMARY OF RESULTS.

Volume of Gastric Secretion.

Subcutaneous injection of pilocarpine nitrate (6·0 mgm.) was quickly followed by abundant secretion into the stomach of a clear opalescent fluid, similar in appearance and usually nearly equal in volume to that produced by histamine (0·25 mgm.). The average 10-minute volume, excluding those cases with achlorhydria, was 24·8 c.c. with pilocarpine, as compared with 23·5 c.c. after histamine. The greatest 10-minute volume was 84 c.c. in the case of histamine and 65 c.c. in the case of pilocarpine. In the majority of cases a maximum volume was reached within 20 minutes of the stimulus, whether by histamine or pilocarpine, after which the secretion gradually returned to its original level.

A second and equal dose of histamine had an almost identical effect to the first, with a tendency for a maximum to be reached a little more quickly. The response to pilocarpine was likewise fairly constant in any one case, with an average value slightly greater after the second than after the first dose.

Histamine and pilocarpine together produced a greater response than histamine alone, the average increase being about 50 per cent.

In cases of pernicious anæmia histamine had no effect, and an almost negligible effect in the remaining cases of achlorhydria (compare Wilkinson, 1932). Pilocarpine, on the other hand, produced a definite response in the majority of the former and in all the latter, in some cases the resultant volume being very considerable.

Gastric Acidity.

The hydrochloric acid concentration increased rapidly after histamine and in most cases very considerably, a maximum being reached in many of the patients within 30 minutes, but in the greatest number of cases this was attained within 20 minutes. With few exceptions there was then a falling off in the acidity, towards, or as far as, the basal level. The highest acidity in any case was 145·6 units, corresponding with a concentration of 0·52 per cent. hydrochloric acid. The output response to a second dose of histamine was similar to or slightly greater than the first, with a tendency for the maximum concentration to be reached more quickly in the second period.

Pilocarpine, on the other hand, showed a marked contrast to histamine, and resulted in an extensive fall in the hydrochloric acid concentration, not infrequently to such an extent that there was little or no free acid in the resulting secretion. The concentration of acid occasionally remained at this level, or gradually increased again in the course of the following hour. Not only was the concentration much reduced, but in every case the total output of acid was very much less after pilocarpine as compared with that following the administration of histamine. The latter, on an average, contained 3·5 times as much acid as the former. Employing a repeated dose of pilocarpine,

there was little difference between the first and second responses, but it appeared probable that for equal volumes of juice, there was rather less acid in the second period.

After histamine and pilocarpine administered simultaneously there was a varying response. On the one hand, the output of hydrochloric acid was greater than after histamine alone; this occurred in nearly half the subjects, and it appeared as though histamine exerted its usual action, being augmented to some extent by pilocarpine. In the remainder of cases, however, the acidity output was less than after histamine alone, and apparently pilocarpine had a relatively greater influence than histamine.

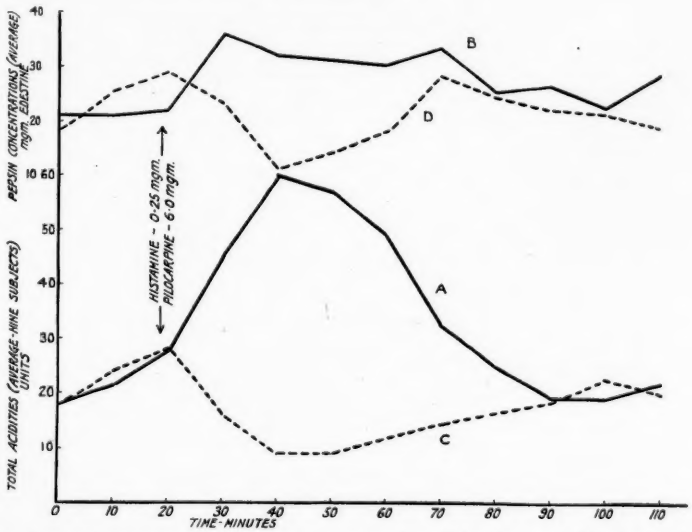

FIG. 1.—Average concentrations of pepsin and total acidities for each 10-minute period secreted under the influence of histamine and pilocarpine. ———— After histamine; (A) Total acidities; (B) pepsin. - - - - - - - After pilocarpine: (C) total acidities; (D) pepsin.

In the achlorhydric group neither histamine nor pilocarpine had any definite stimulatory effect on the acidity.

Pepsin.

The administration of histamine in almost every case was followed immediately by an increased *output* of pepsin. A second dose of histamine had, on the whole, a slightly less effect, although in one case only the second dose effected an increase. The *concentration* was either increased or decreased—more often the former.

Pilocarpine usually produced an increase in the output; but this was nearly always less than with histamine, and in a few cases the output was decreased after pilocarpine. The concentration was more often decreased than increased.

and was lower than the corresponding concentration with histamine. A second dose had an effect similar to that produced by the first, with a tendency for the second response to be slightly less than the initial one as regards the average output. Histamine and pilocarpine together generally resulted in a slightly greater output than after histamine alone.

In two of the cases of achlorhydria, not associated with pernicious anæmia, histamine produced a slight increase in the pepsin content, but in the remainder the pepsin was negligible both after pilocarpine and after histamine.

The results are conveniently illustrated by the 9 cases in Table I and Fig. 1. The Table shows the average 10-minute volume, *output* of total acid, and *output* of pepsin respectively of all the samples (taken over 1½ hours) in each of 9 subjects that received histamine and pilocarpine separately on different days. In the figure, however, a direct comparison is made of the influence of histamine and pilocarpine separately, but the curves represent the *average concentrations* of all the corresponding 10-minute samples of these same 9 individuals. They are self-explanatory.

TABLE I.—*Effect of Histamine and Pilocarpine on Gastric Secretion.*

Case No.	Total acidity. Average output per 10-minute period—units.		Average volume per 10-minute period.		Pepsin. Average number mgm. edestine digested per unit time by each 10-minute volume.	
	After histamine.	After pilocarpine.	After histamine.	After pilocarpine.	After histamine.	After pilocarpine.
12	2·0	1·1	12·5	22·0	2·7	1·9
28	3·8	1·5	9·5	18·2	3·3	3·9
31	20·2	2·6	29·5	25·1	7·3	8·6
32	2·6	0·7	11·3	21·2	2·1	0·0
33	8·7	1·6	14·9	7·8	0·0	0·0
34	8·8	5·2	17·7	26·4	6·8	9·0
35	3·4	2·7	8·5	16·5	2·8	2·9
36	4·8	2·4	12·9	12·6	3·4	1·5
37	4·1	1·5	6·7	7·0	2·0	1·3

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OBSERVATIONS UPON THE ABSORPTION OF CALCIUM IN NORMAL ANIMALS.

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THE opinion is very generally held that irradiated ergosterol increases the absorption of calcium from the intestinal tract of normal animals (Clouse, 1932 ; Chick, 1932 ; Harris, 1932 ; Harris and Innes, 1931). Yet, as a matter of fact, little direct evidence can be adduced for the support of this view. The restoration of normal calcification in rickety animals, as a result of ergosterol treatment, the conversion of a calcium balance from negative to positive by treatment with cod-liver oil or by irradiation with ultra-violet light, and the greater hypercalcæmic and other toxic effects of irradiated ergosterol upon a high than upon a low calcium diet, are among the observations which have led to the conclusion that the absorption of calcium is increased in non-rachitic animals by ergosterol administration. Our experiments, however, give no indication that the sterol causes this effect.

That the hypercalcæmia induced by overdosage with irradiated ergosterol is not due to increased absorption of calcium but is the result of the withdrawal of the mineral from the skeleton seems definite enough. In previous experiments from this laboratory (Taylor and Weld, 1931) it was shown that hypercalcæmia and the other toxic mainfestations of overdosage could be produced by the administration of irradiated ergosterol to dogs in negative calcium balance. Hypercalcæmia has also been produced in dogs (Hess *et al.*, 1931 ; Jones and Rapoport, 1931) fed upon calcium-free diets.

It was also pointed out in a previous communication (1932) that the reduction in fæcal calcium which occurs toward the end of the overdosage condition was not due to increased absorption, but to the depression of the normal excretory function of the bowel wall for calcium. Harris and Innes, realizing the difficulty of distinguishing the excreted from the non-absorbed calcium, refer to the difference between the quantity of the mineral ingested and that appearing in the fæces as the " net absorption ". The absorption of calcium is an important practical question, and if the depression of the excretion of calcium were brought about by therapeutic doses of irradiated ergosterol, and were unaccompanied by an increased loss of calcium in the urine, then reduced excretion through the bowel would indicate increased retention by the body, and probably a beneficial reaction to the administration of irradiated ergosterol. We have found, however, that in dogs the depression of the excretion of calcium through the bowel-wall is purely an overdosage phenomenon, and is more than balanced by the increased excretion of calcium in the urine which occurs at about the same time. At the time that the depression of calcium excretion through the bowel-wall occurs, the net result to the body is a loss rather than a gain in calcium, and the hypercalcæmia of ergosterol

overdosage therefore cannot be explained either by increased absorption, or by reduced excretion of calcium through the gut. The experiments about to be reported also show clearly, we believe, that *small* (therapeutic) doses of irradiated ergosterol do not increase the absorption of calcium in normal animals. We have no data so far upon rachitic animals.

METHODS OF ANALYSIS OF FOOD AND EXCRETA.

The dogs were kept in metabolism cages and all the urine and fæces collected throughout the period of observation. Twenty or 30 gm. of infusorial earth were added to the daily diet of each dog in order to ensure as far as possible a formed movement. At first collections and analyses were made daily, but in the later experiments analyses were done weekly upon the pooled collections. The urines were kept strongly acid and 50 c.c. aliquots were dried and ashed, the ash well mixed in a mortar, and an aliquot, consisting of about a quarter of the whole, was taken by weight. This was boiled for 5 minutes in diluted nitric acid and the undissolved residue filtered off and washed with water. Further extractions of this material yielded no appreciable quantity of calcium.

The solutions were then neutralized with strong ammonia, using rosolic acid as an indicator, and re-acidified with 8 drops of concentrated HCl. The solution was then heated, and 10 c.c. of 2·5 per cent. oxalic acid added while boiling. After cooling, 8 c.c. of 20 per cent. sodium acetate were added, the solution changing colour from a light to a dark yellow or tan. After standing overnight the precipitate was filtered, washed twice with a saturated solution of calcium oxalate and twice with distilled water. Then, after perforating the paper, the precipitate was washed off the paper first with water and afterward with hot N/1 sulphuric acid. Two c.c. of concentrated H_2SO_4 were then added to dissolve the precipitate, and the solution titrated while hot (60°–80°) with N/10 potassium permanganate until a faint permanent (1 minute) pink colour remained.

Titrating in this way, 1 c.c. of the permanganate is equivalent to 2 mgm. calcium. In every case, enough of the original ash solution was taken to require a final titration of 10–20 c.c. of the permanganate.

The food substances were analysed in a similar way, that is, suitable portions were ashed and the ash treated in the manner described. Non-spoiling foods, such as dog biscuit and bread, were dried and ground up, and a sufficient amount prepared to last for months. Perishable foods, such as meat, were analysed frequently, and were found to be fairly consistent in their calcium content. The calcium salts added to the diet were also analysed and the amounts given were based on this analysis. Different lots of calcium lactate were found to differ greatly in their calcium content.

Every food portion was weighed, and no trouble was experienced in ensuring that the whole was eaten. The drinking water was distilled.

From time to time a day's ration was analysed in order to prove that the calculated calcium content was correct, and it was always found so. At frequent intervals a specimen (usually of fæces or food) was also determined by a standard gravimetric procedure, and on several occasions specimens were submitted to other laboratories for independent analyses.

EXPERIMENTAL.

The Absorption of Extra Calcium by Normal Dogs.

In a preliminary series of experiments already reported (1932) it was found that the calcium of an ordinary diet was completely absorbed. Whether the animals were starved or upon a diet of meat and biscuit or were given moderate amounts of extra calcium in the form of calcium chloride, the calcium content of the fæces remained unchanged. The calcium of the fæces under these circumstances must obviously have been excreted from the blood into the bowel lumen.

The greatest amount of calcium received by the animals reported in the previous paper was 146 mgm. daily. In the present experiments the animals were fed with extra calcium in gradually increasing amounts over a period of several months. In order to obtain the true relationship between the calcium of the fæces and the calcium ingested, and to avoid inaccuracies resulting from the overlapping of periods, the time over which a given dose of calcium was administered was in no instance permitted to be shorter than 10 days, and was usually much longer. In any event, the period was long enough to allow the fæcal calcium to reach an approximately steady level. One animal, Dog No. 54, has been under observation for 14 months ; another, Dog No. 81, for 8 months ; a third, Dog No. 104, for 12 months ; and others for 3 and 4 months.

Eight animals, Dogs Nos. 54, 72, 81, 94, 104 and 115 and Puppies I and K, have been employed in this series of experiments. The extra calcium was given in the form of the chloride to Dogs Nos. 54 and 72, as the lactate to Dogs Nos. 81, 104 and 115, as the gluconate to Dog No. 94 and as the phosphate to I and K. The experiment upon Dog No. 54 (weight 20 kilo) was started by putting it upon a diet of meat and biscuit. The animal was continued for 4 weeks upon this diet, of which the daily ration contained 78 mgm. of calcium. The average daily quantity of calcium passed in the fæces during the first half of the period was 12 mgm., and 30 mgm. during the second half. During the fifth week the animal was starved. The fæcal excretion remained practically unchanged. Since the calcium of the fæces during the fifth week must have been excreted calcium, it may be concluded that the calcium of the diet fed during the previous 4 weeks had been completely absorbed. In subsequent periods extra calcium was given in the form of a solution of calcium chloride in gradually increasing amounts. No important increase in the fæcal calcium resulted until the quantity ingested each day had been raised to nearly 3 gm. It can be seen from Fig. 1 that an increase of the ingested calcium from 78 mgm. to nearly 3 gm. daily caused an average daily increase in the fæcal calcium during the two weekly period of only about 280 mgm. Continuation of the administration of calcium at the above level, however, caused the calcium of the fæces gradually to increase. The difference between the amount of calcium ingested and that passed in the fæces, *i. e.* the absorbed calcium, consequently diminished (Fig. 2), Nevertheless, absorption continued at a high value for the next 4 weeks. From the commencement of the experiment to the end of this period some 150 gm. of calcium had been ingested ; of this 116 gm. had been absorbed. After this time the plan of the experiment was altered by the administration of irradiated ergosterol. The results of this administration will be considered in a later section.

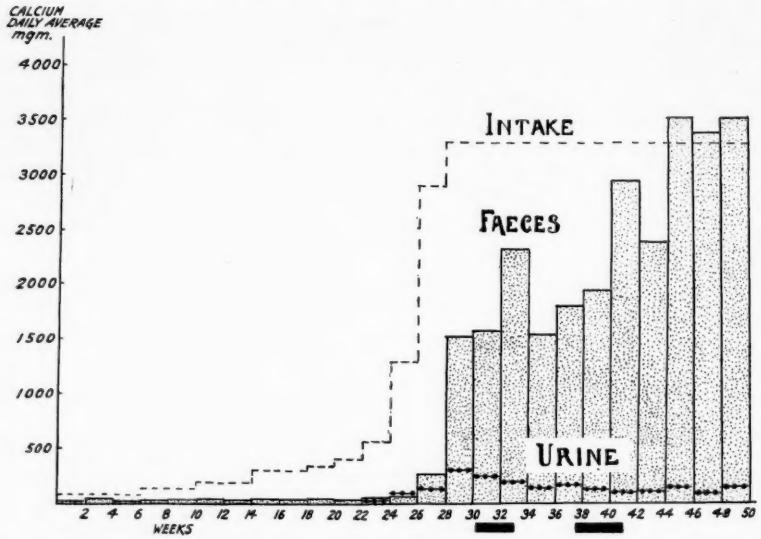

FIG. 1.—Curves showing the calcium excretion of Dog No. 54 following the administration of extra calcium. Black bars indicate periods of ergosterol treatment.

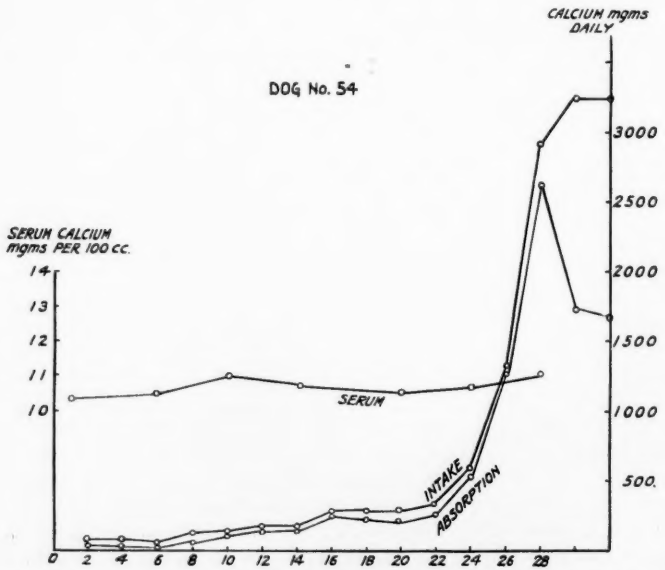

FIG. 2.—Curves of calcium intake and absorption and of serum calcium in an experiment upon Dog No. 54.

The urinary calcium showed no change until the ingested calcium had reached a level of a gramme or more, when it increased by about 100 per cent. This occurred before any increase in the fæcal calcium was observed. The figures for the urinary calcium in subsequent periods (22nd to 50th week) are shown graphically in Fig. 1. In no period did the daily average of the urinary calcium exceed 350 mgm. No rise in the serum calcium was observed,

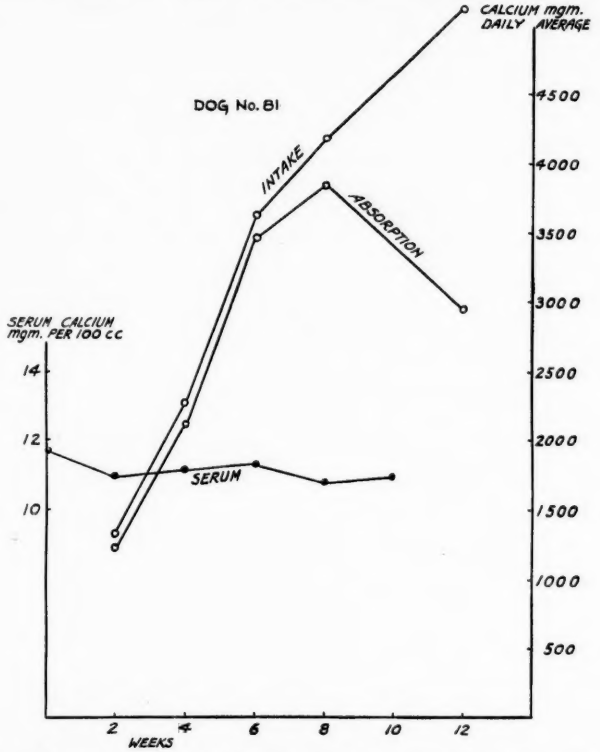

FIG. 3.—Curves of calcium intake and absorption and of serum calcium in an experiment upon Dog No. 81.

except in some instances in which a small temporary one was seen about 2 or 3 hours after the extra calcium had been fed.

Similar results to those described for Dog No. 54 were obtained in experiments upon Dog No. 81. This animal was given the calcium in the form of the lactate, and the experiment was commenced with much larger doses than those given to dog No 54. Little change in the fæcal calcium became evident until the dose was raised to over $3\frac{1}{2}$ gm. of calcium per day. Throughout the entire period of observation (nearly 8 months) 1·225 gm. of calcium were fed; of this, 616 gm. were absorbed (Fig. 3). The results of the foregoing series of

experiments are in agreement with those of Sherman and Hawley (1925), who showed that increasing the calcium content of the diet of children increased the absorption. The results obtained upon Dogs Nos. 104 and 115 are interesting in that these animals were in pronounced negative calcium balance for some 10 weeks before the extra calcium was given. The administration of about a gramme of calcium in the form of the lactate reversed the balances. The experiments with calcium gluconate and tricalcium phosphate showed that large quantities of calcium were absorbed when given in either of these forms. The absorption of calcium given as the phosphate, though large at first, did not maintain a high level for as long a time as when either the chloride, lactate or gluconate was employed.

The Effect of Irradiated Ergosterol upon the Excretion of Calcium through the Bowel Wall.

The effect of irradiated ergosterol in massive doses in depressing the normal excretion of calcium through the bowel is very definite. We have observed this to occur, however, only in the later stages of the overdosage condition, and, as already mentioned, the urine excretion at the same time is increased to an extent which more than counterbalances the tendency toward the retention of calcium within the body which the depression of intestinal excretion appears to imply. We have never been inclined to draw the conclusion from this effect of overdosage that a similar result would follow the action of ergosterol in therapeutic dosage. But since such an action has been suggested by others, we decided to put the question to the test of the following experiments (Table I).

TABLE I.—*The Results of the Administration of Small Doses of Irradiated Ergosterol to Dogs in Negative Calcium Balance.*

Consecutive 2-weekly periods.	Dog No. 104. Food contains 75 mgm. Ca daily. Fæces mgm. Ca daily average.	Dog. No. 132. Food contains 10 mgm. Ca daily. Fæces mgm. Ca daily average.	Dog. No. 134. Food contains 10 mgm. Ca daily. Fæces mgm. Ca daily average.	
1st	157	451	400	Control periods.
2nd	316	619	437	
3rd	333	316	430	
4th	466	312	447	Periods of ergosterol administration.
5th	333	428	486	
6th	223	520	734	

Dogs Nos. 104, 132 and 134 were placed upon low calcium diets. The diets of Nos. 132 and 134 were practically calcium-free, the daily ration containing only 10 mgm. The calcium of the fæces was determined upon these animals for a perliminary period of six weeks. During this time all three were in pronounced negative calcium balance. The total quantity of calcium passed in the fæces during this preliminary period was several times greater than the quantity of calcium taken in the food. The great bulk of the fæcal calcium

must, therefore, have been excreted calcium. At the end of the control period irradiated ergosterol was given in therapeutic dosage (0·06 c.c. of 250 D per kg.). No significant reduction in the fæcal calcium occurred. It may, therefore be concluded that the ergosterol administration did not depress the excretion of calcium by the intestinal epithelium.

The results of the experiments described in the preceding sections have, we believe, a direct bearing upon the question raised in the first paragraph of this paper, namely, whether or not the administration of irradiated ergosterol to normal animals has an influence upon calcium absorption. Our experiments show that a normal dog absorbs all the calcium contained in an ordinary diet, and that not until the amount of ingested calcium far exceeds that which could ever be taken as a constituent of the food does a failure in absorption occur. The large quantities of calcium absorbed, be it noted, caused at the most a relatively slight and very temporary rise in the serum calcium. These observations offer further evidence against the view that the hypercalcæmia following ergosterol overdosage is the result of increased absorption from the intestinal tract.

It also seems perfectly clear that therapeutic doses of irradiated ergosterol do not increase the absorption of calcium in normal animals. Since the bowel is capable of absorbing such large quantities of calcium, it is evident that the calcium contained in any ordinary diet will be completely absorbed. Irradiated ergosterol, therefore, could have no effect upon the absorption. On the other hand, when the diet is low in calcium and the animal is in negative calcium balance, irradiated ergosterol does not depress the excretion of calcium through the bowel wall. The negative balance is not reduced. Furthermore, increased absorption of calcium can be induced by increasing the calcium intake without the aid of irradiated ergosterol, and, as shown in Dogs Nos. 104 and 115, large quantities of added calcium are absorbed by, and a positive balance induced in, an animal which previously had been in pronounced negative balance. The administration of ergosterol would therefore appear to be of little benefit so far as the " net absorption " of calcium in normal animals is concerned.

The Administration of Irradiated Ergosterol to Animals Receiving Large Quantities of Extra Calcium.

Dog No. 54 was given 0·1 c.c. (0·05 c.c. per kilo) of irradiated ergosterol (250 D.) daily from April 26th to May 13th, 1932, and from June 13th to July 11th (Fig. 1). This animal had been placed upon a calcium intake of over 3 gm. 5 weeks prior to the first ergosterol period and was continued upon this throughout both ergosterol periods. As will be seen from the chart, Fig. 1, there may have been a tendency for the fæcal calcium to rise gradually in the periods following later upon the high calcium intake, yet it is suggestive and seems to be more than accidental that the fæcal calcium during the ergosterol periods rises well above the general trend of the curve. This is especially noticeable during the latter part of and immediately following the first ergosterol period, for, apart from the high level of the fæcal calcium coincident with the time during which the administered ergosterol might, from our experience of the delayed action of this material, be expected to exert its effect, the calcium

output remains at a steady level. At any rate there is no indication that the sterol has decreased the calcium of the fæces. On September 26th the high calcium feeding was discontinued. The animal for the succeeding 6 weeks received only 78 mg. of calcium daily. During this period a fall occurred in the fæcal calcium. At the end of the sixth week, at which time the calcium of the fæces had reached a low level, ergosterol was administered for two weeks in the same daily dosage as in the previous periods. The low calcium diet was continued. A rise in the fæcal calcium resulted. In Fig. 4 the fæcal

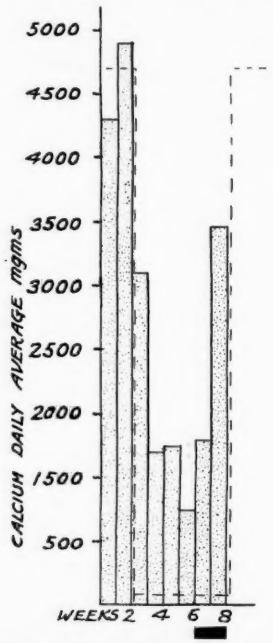

FIG. 4.—Effect of the administration of irradiated ergosterol upon the fæcal calcium of Dog No. 54. Stippled blocks = fæcal calcium; broken line = calcium intake; black bar = ergosterol administration.

calcium during this period of ergosterol feeding is shown in weekly periods. Similarly suggestive results were obtained in experiments upon Dog No. 81.

In another experiment the absorption of added calcium was compared in two animals, Nos. 104 and 115, one of which received small doses of ergosterol. Determinations of the fæcal calcium had been made upon these animals for several weeks. Though the excretion of No. 104 was somewhat less than that of No. 115, nevertheless, the two animals were comparable as to diet and general conditions. The calcium of the diet was low to start with, but after a period of 8 weeks this was more than doubled for each animal. In the latter two weeks of the period of low calcium intake and throughout the period of increased intake Dog No. 104 alone received small doses of irradiated ergosterol.

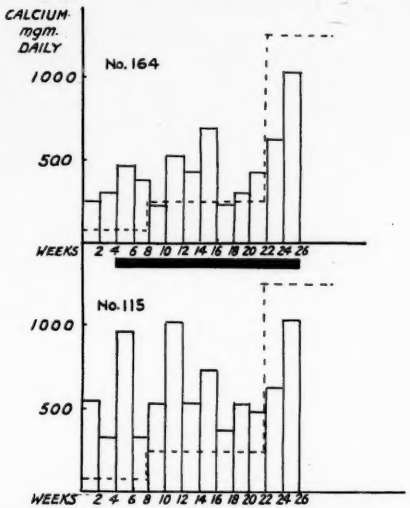

FIG. 5.—Curves of calcium intake and fæcal calcium of Dogs Nos. 104 and 115. Blocks indicate fæcal calcium, broken line calcium intake and black bar ergosterol administration.

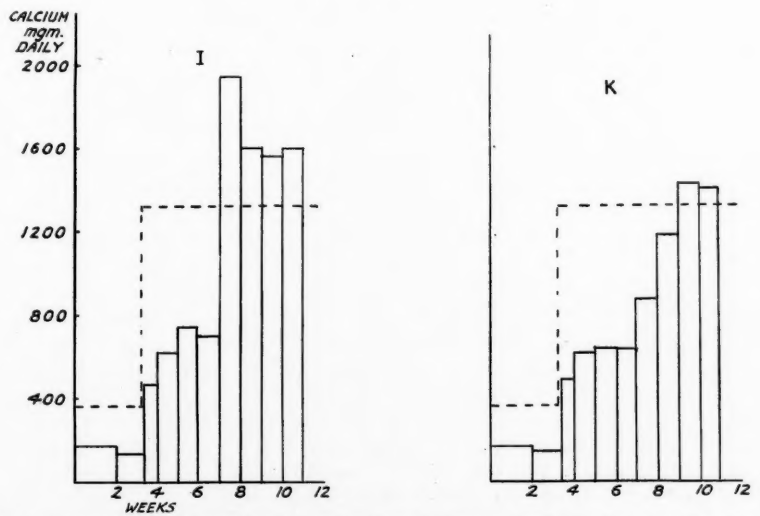

FIG. 6.—Curves of calcium intake and fæcal calcium of Puppies I and K. I received 0·06 c.c. ergosterol (250 D.) per kilo daily throughout the experiment. Blocks indicate calcium of fæces, broken line calcium of the food.

There is a very slight increase in the fæcal calcium of Dog No. 115 as a result of the increased intake. Dog No. 104 shows a relatively greater increase of fæcal calcium—that is, the added calcium is not absorbed more readily, but indeed relatively less so, apparently, by the ergosterol-treated animal (Fig. 5). Upon increasing the calcium intake of each animal to about 1200 mgm. the fæcal calcium rose to about the same extent in both, though No. 104 was still receiving daily doses of ergosterol. An experiment of the same nature was carried out upon two puppies, I and K. One (I) received 0·06 c.c. of ergosterol (250 D.) per kg. and the other received none. The excretion of both on a low calcium diet was of practically identical value, but when extra calcium (Ca_3Po_4) was fed, considerably less, actually, was absorbed by the treated animal (Fig. 6).

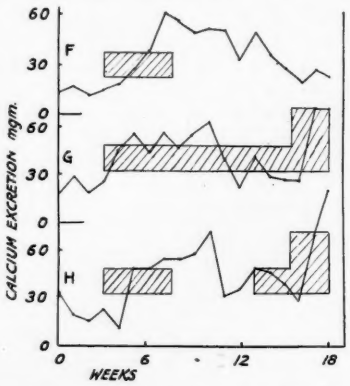

FIG. 7.—Curves showing the calcium excretion of the three Puppies F, G and H. The hatched blocks represent periods of ergosterol treatment. See text for the details of this experiment.

The Effect of Irradiated Ergosterol upon the Calcium Excretion of Puppies.

Three puppies F. G and H, of the same litter and about three months of age, were placed upon a diet consisting of meat, bread and milk. The three animals were kept upon this diet for 4 weeks. The urine and fæces were not collected separately, calcium determinations being made daily upon the combined excreta. At the end of the 4-week period therapeutic doses of irradiated ergosterol were given daily to each animal and continued for over 7 weeks. No change was made in the diet during this time. The curve of fæcal excretion rose steadily throughout this period in Puppies F and G. The calcium excretion of H showed some reduction during the first week of the ergosterol administration, but rose rapidly in the following week and remained high thereafter. Though there was no altered condition other than the administration of ergosterol which might account for the rise in the calcium excretion of these animals, it is possible that such existed, and unfortunately a control animal was not run at this time. However, it can be said at least that no increase in calcium absorption during the administration of the ergosterol was demonstrable (Fig. 7). After a little more than 7 weeks the administration to Puppies F and H, but not to G, was discontinued. At the end of the

thirteenth week H was given ergosterol in the same dosage as that employed with G. This dose was continued for over two weeks. At the end of this time the dose given to each of these two animals was increased two and a half times. The curves of calcium excretion show a considerable degree of fluctuation, but all three exhibit a remarkable parallelism, except toward the end of the experiment, when, upon increasing the dose of ergosterol, a prompt increase in the calcium excretion of G and H, but not of the untreated animal F, occurs.

The effect of ergosterol administration upon the fæcal calcium was studied in three puppies of another litter, I, J and K. The daily calcium intake was 98 mgm. All three animals were run for a preliminary period of 6 weeks, calcium determinations being made daily. At the end of this time 0·06 c.c. irradiated ergosterol (250 D.) per kilogramme of body-weight was given daily to two of these animals. K was kept as a control. In all three animals the calcium excretion rose throughout the period of the experiment, but no important difference between the calcium excretion of the ergosterol-treated animals and that of the control was observed (Fig. 8). It may be noted that I and J passed into negative calcium balance before the control animal K. All three animals showed identical growth curves.

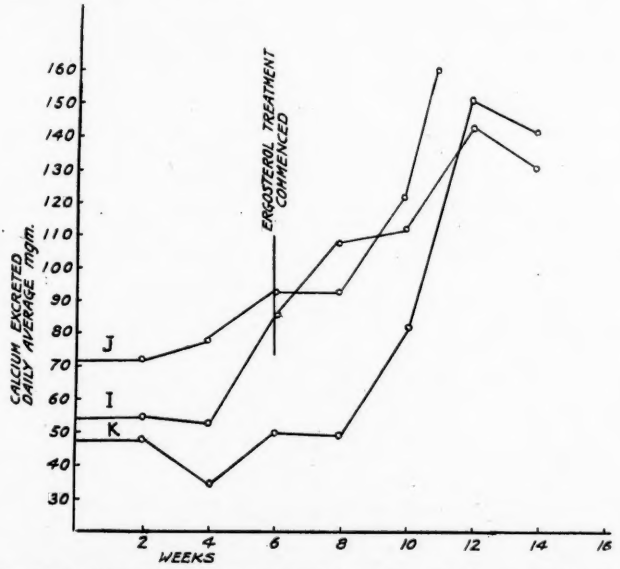

FIG. 8.—Curves of calcium excretion in Puppies I, J and K. The broken vertical line cutting the upper two curves indicates the commencement of ergosterol treatment, which was continued to the end of the experiment.

SUMMARY AND DISCUSSION.

The results of the experiments described in this paper indicate that in normal animals the calcium contained in the diet is completely absorbed. Even when

extra calcium is given, the calcium excretion does not increase until the intake reaches a high figure. Though overdosage with irradiated ergosterol depresses the excretion of calcium through the bowel, small doses do not exert this effect, and there was no indication in any of our experiments that the net absorption of calcium was increased by the administration of the sterol in therapeutic dosage. Though high calcium feeding will convert a negative calcium balance to positive, such a result has not been observed as a result of ergosterol administration, and when an animal is on a high calcium intake the administration of ergosterol does not increase the quantity of calcium absorbed. The results seem to point to the sterol diminishing rather than increasing the net absorption of calcium.

Several workers (Harris, 1930; Bills and Wirick, 1931) have reported that a high calcium diet increases the hypercalcæmia and other toxic effects of irradiated ergosterol, and Mellanby (1921) and others have found that the calcifying action of vitamin D is enhanced by a high calcium diet. Such observations seem to have advanced the belief that irradiated ergosterol, either in therapeutic or excessive doses, increases calcium absorption in normal animals. The action of calcium in augmenting the overdosage effects of ergosterol may be explained without assuming that the latter encourages calcium absorption. A high calcium intake also magnifies the effects of parathormone, yet it has never been suggested that this substance has any influence upon absorption. Overdosage with either parathormone or irradiated ergosterol causes the withdrawal of calcium from the skeleton. It is reasonable to assume, then, that the calcium absorbed from the intestinal tract will fail to be deposited in the bones at such a time that the calcium stream, as a result of either of these substances, is flowing from bones to blood. The calcium, which our experiments show is absorbed under any circumstances, would be thereby barred from entering the skeletal storehouses, and a rise in the serum calcium and increase in its excretion in the urine would follow inevitably. This explanation of the action of calcium in enhancing the overdosage effects of ergosterol is more in accord with the known facts than the one which assumes an increased calcium absorption.

We wish to extend to Dr. C. H. Best our cordial thanks for the interest he has shown in these researches.

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